Changes in *Lepeophtheirus salmonis* gene expression during host switching and selection

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ABSTRACT

*Lepeophtheirus salmonis* is a copepod parasite of salmonid fish, commonly referred to as sea lice, and is particularly pathogenic to Atlantic salmon (*Salmo salar*). Although endemic on wild salmon, *L. salmonis* infection intensity rarely reaches clinical disease levels in wild fish. The stationary nature and high density stocking of Atlantic salmon in marine aquaculture allows the intensity of *L. salmonis* infection to reach clinical disease levels. In certain areas, during high levels of infection, salmon often develop gross lesions, most commonly on the head, dorsal and perianal regions. The lesions, coupled with the stress induced by infection, can result in decreased feeding, secondary infections and mortality of fish. Over the past 40 years a number of chemotherapeutants have been used to control *L. salmonis* infection in farmed fish, however, reliance on a relatively small number of treatments has driven the development of drug resistant populations of *L. salmonis.*

Due to the high cost of developing new drugs, whose use may be limited by resistance development or environmental impacts, development of alternative treatment methods is becoming a necessary research avenue. Preliminary studies have shown that release of compounds derived from non-salmonid fish can reduce infection of salmon. The content of this thesis has the dual goal of trying to elucidate some of the behaviours of adult *L. salmonis* during host switching, and the development of an *in vitro* assay to assess the effects of the anti-attachment compound allyl isothiocyanate.

Host switching behaviour was assessed using a cohabitation of *L. salmonis* infected and uninfected fish. It was found that male lice reached an equal distribution between the two populations of fish, while female lice did not switch hosts. This equal distribution of male lice is suggested to increase access of each lice to food and potential mates. Eight genes associated

with various facets of lice survival underwent gene expression analysis (CYP18 A1-like gene, Trypsin-1, tissue plasminogen activator precursor-like (TPAP)-like gene, cyptochrome p450 isoform 1-like gene, peroxinectin-like gene, leukocyte receptor cluster member 9-like (LRCM9) gene, glycene receptor α-2-like gene, and a nicotinic acetylcholine receptor subunit-like (nAChR) gene) and five salmon genes associated with immune status were analysed (Interleukin (IL)-1β, IL-8, IL-12, IgT, and Matrix Metalloproteinase 9 (MMP9)) in an attempt to reveal patterns in gene expression during host colonization. Only one instance of significant difference occurred during the study, between MMP9 in the spleen of initially infected salmon and the spleen of initially uninfected salmon at 2 days post cohabitation. It was thought that the variability in gene expression of individual lice and fish that accompanied the use of outbred populations may have masked potential changes in gene expression. Pearson correlations were used to compare gene expression between individual lice and its respective host; expression of a peroxinectin-like gene had several instances of significant correlation with expression of host genes. This is thought to be caused either by peroxinectin-like having a role in modulation of host immune response, or by peroxinectin-like being expressed in response to the salmon reaction to infection. It appears that fish with low infection have more instances of significant correlation with several *L. salmonis* than fish with high infection levels.

*In vitro* bioassays designed to assess lice response to allyl isothiocyanate revealed that adult and copepodid lice experienced increased mortality in higher treatment doses (>1ppm). Gene expression of 7 genes associated with different facets of survival were analyzed for dose dependent effects of allyl isothiocyanate on *L. salmonis*. The outcomes of this study suggests allyl isothiocyanate causes a significant amount of stress to *L. salmonis*, possibly causing the initiation of an immune response (increased then decreased LRCM9-like gene), reduced feeding (decreased Trypsin-1), and a nociceptive response (increased nAChR-like gene). Immobility was observed in lice at the high dose treatment, which is not necessarily one of the intended endpoints and may not be an achievable concentration in any management strategy, but provides evidence to suggest that exposure to allyl isothiocyanate may induce avoidance behaviour, reduce potential settlement on the host, and ultimately have a negative impact on lice survival.

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**List of Abbreviations**

| | |
|---|---|
| ABMA | Aquaculture Bay Management Areas |
| BKD | Bacterial kidney disease |
| cT | Threshold cycle |
| DNA | Deoxyribonucleic acid |
| Dpc | Days post cohabitation |
| EF-1α | Translation eukaryotic elongation factor 1α |
| GAPDH | Glyceraldehydes-3-phosphate dehydrogenase |
| GRα-2 | Glycene receptor α-2 |
| IgT | Immunoglobulin T |
| IL | Interleukin |
| LPS | Lipopolysaccharide |
| LRCM9 | Leukocyte receptor cluster member 9 |
| MMP9 | Matrix Metalloproteinase 9 |
| MNRQ | Mean normalized relative quantity |
| nAChR | Nicotinic acetylcholine receptor |
| PCR | Polymerase chain reaction |
| Pearson C. | Pearson correlation |
| $PGE_2$ | Prostaglandin E2 |
| P-gp | P-glycoprotein |
| Ppb | Parts per billion |
| Ppm | Parts per million |
| Ppt | Parts per thousand |

| | |
|---|---|
| qPCR | Quantitative polymerase chain reaction |
| RNA | Ribonucleic acid |
| RPS20 | Structural ribosomal protein S20 |
| RQI | RNA Quality Indicator |
| SEM | Standard error of the mean |
| TMS | Tricaine methanesulfonate |
| TPAP | Tissue plasminogen activator precursor |

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# <u>Chapter 1</u>

**GENERAL INTRODUCTION TO ATLANTIC SALMON AND *LEPEOPHTHEIRUS SALMONIS***

## 1.1 Atlantic salmon (*Salmo salar*)

Atlantic salmon are an anadromous fish species, meaning they spawn and spend their first few years living in fresh water before migrating to sea to grow and mature. They are found in the North Atlantic Ocean, Baltic Sea, and North Sea and historically have been found in all countries with rivers draining into any of these bodies of marine water. Atlantic salmon are important economically and culturally in many of these countries (Verspoor, Stradmeyer, and Nielsen 2007).

There are seven stages in the Atlantic salmon lifecycle. Once hatched, the young fish are termed alevin, are completely dependent on their yolk sac for nourishment, and remain in the gravel nest built by their mother. The fry stage follows alevin, and they remain in the nest, but do not rely on their yolk as a primary source of food, eating food that enters the redd from the water. Once dispersed from the nest, fry mature into parr and begin feeding on food in the water. They will remain in fresh water for approximately 4 years before migrating downstream to the ocean. Parr may alternatively mature in fresh water, never migrating to the ocean. Mature adult fish will spend 1-4 years at sea, feeding and growing, before returning to their native stream and spawning. Once spawned, the majority of fish will perish, but approximately 5% will return to the ocean, recover, and spawn a second time. Between spawning and return to the ocean the fish are termed kelt (Shearer 1992). Salmon found in the inner Bay of Fundy are atypical, spending 2 years as parr, and typically only heading to marine waters for one winter before returning to rivers to spawn. Additionally, this population of fish is more likely to have repeat spawners (Verspoor, Stradmeyer, and Nielsen 2007).

### 1.1.1 The decline of Atlantic salmon

As early as 1930 there was a noted decline in the numbers of wild salmon in eastern Canada and the United States, with extirpation occurring in several regions including the Penobscot and Kennebec rivers. At this time it was speculated that there were three driving factors behind the decline of salmon numbers: restriction of free passage up rivers to the spawning grounds, pollution and habitat disruption, and unsustainable catch rates (Calderwood 1930). A report released by the Provincial Atlantic Salmon Working Group also identified habitat disruption and unsustainable fishing as major contributors to the decline in salmon stocks (1986). In 1985 New Brunswick, Nova Scotia, Prince Edward Island, parts of Quebec and Newfoundland eliminated the commercial harvest of Atlantic salmon in an effort to preserve remaining salmon stocks. In this same time period, recreational anglers were given restrictions on the number and age of fish they were allowed to catch (Special Federal/Provincial Atlantic Salmon Working Group 1986). Today, the commercial wild Atlantic salmon fishery remains closed in Canada, and recreational fishing is closed in many rivers and highly restricted in others in an effort to preserve remaining fish populations (Department of Fisheries and Oceans Canada 2011b, 2012b).

### 1.1.2 The rise of Atlantic salmon aquaculture in the Atlantic Canada

Atlantic salmon is a highly desired food product and the production/harvest of salmon has been an important source of employment in small communities. Development of salmon hatchery programs to replenish wild fish stocks became a popular tool used to help maintain wild populations (Alder Fork Consulting 2001). Hatchery replenishment of wild populations failed to recover salmon numbers to abundance where the commercial fishery could operate in Canada. The consumer demand for Atlantic salmon continued to exist, and in the 1970s the first experiments with sea cage farming of Atlantic salmon in Canada took place. The first commercial success of the Canadian industry took place in the 1980s (Department of Fisheries and Oceans Canada 2013a). Today, Atlantic Canada produces 32, 000 tonnes of salmon annually, with a value of $192 million (Aquaculture Sustainability Reporting Initiative 2012).

In Atlantic Canada, Atlantic salmon are primarily farmed in New Brunswick, with a growing number of farms in Nova Scotia and Newfoundland. Several salmon species are also cultured in British Columbia, Canada (Canadian Aquaculture Industry Alliance 2012). Eggs and milt are collected from mature fish (cultured broodstock), and used to generate fertilized eggs. Once hatched, fish are typically raised in an indoor, freshwater facility until the fish smolt, approximately 6-12 months after egg fertilization. Smolts are transported to sea cages, located on a tenure lease in ocean waters. Sea cages are floating containment structures, typically being a circular frame approximately 30m in diameter, which supports a net approximately 10m deep. The openness of the net allows new water to pass through the sea cage with the currents, providing water changes and aeration for the fish. Depending on the farm and time of year, fish are fed a commercial pellet feed daily, until satiation is reached. Cold water temperatures, inclement weather, and disease state may result in fish not being fed for extended periods. Fish are grown to a market size of approximately 10lbs before harvest, typically spending 14-24 months in grow-out. During harvest, fish are pumped out of their cage on to a boat with specialized stunning and storage equipment. The fish are stunned and have their gills slit before being added to a holding container filled with ice. The harvested fish are then transported back to land where they are processed into their final market product (Marine Harvest 2012).

## 1.2 Diseases in Atlantic salmon aquaculture

The open-to-the-environment nature of salmon farming allows for contact between farmed and wild salmon. Salmon farms are often located near suitable wild salmon habitat, as the water conditions needed to maintain the health of aquaculture fish are also amenable to wild salmon. This has the dual effect of introduction of endemic diseases to farms, and the maintenance of disease within the farm. In Atlantic Canada several diseases have been reported on salmon farms, including protozoan, bacterial, viral, fungal and copepod infections, some of which are described in Table 1.1. While many of these diseases are not thought have large impacts on wild fish populations, the stationary nature and high density of farmed fish allow the development of self-sustaining infections that can have a much larger health impact on fish than might naturally occur.

The common salmon diseases (Table 1.1) all have a vaccine available for use as a method of pathogen control, except for the fungabacterial kidney disease (BKD) and *L. salmonis.* In Atlantic Canadian aquaculture prevalence of BKD in affected farms ranges from 1-5%, while the prevalence of *L. salmonis* reaches 100% in farmed fish when untreated (BC Centre for Aquatic Health Sciences 2010; Glover et al. 2004). Unlike BKD, *L. salmonis* cannot be controlled at the hatchery level, as this parasite cannot survive in fresh water and only infects fish once they are transferred into sea cages. This makes the implementation and management of proper husbandry techniques and continued development of effective therapeutic treatments particularly important to mitigate the impacts of *L. salmonis* infection.

**Table 1.1:** Common diseases of farmed Atlantic salmon in Atlantic Canadian waters.

| Disease | Common Name | Impact | Treatment |
|---|---|---|---|
| *Vibrio anguillarum* and *V. salmonicida* | Vibriosis, Cold Water Vibriosis or Hitra Disease | Haemorrhage of internal visceral tissues and at the base of fins, lethargy, mortality | Vaccination and good husbandry |
| *Aeromonas salmonicida* | Furunculosis | Furuncles (boils) on skin/muscle, surface haemorrhages, lethargy, high mortality | Ensuring smolts are disease free, vaccines, improved husbandry, proper site fallowing practices |
| *Renibacterium salmoninarum* | Bacterial Kidney Disease | Protruding eyes, haemorrhage at the base of fins, lethargy, chronic mortality | Eliminating use of infected broodstock |
| *Birnaviridae* family | Infectious Pancreatic Necrosis Virus | White fecal casts, lethargy, mortality, resistance develops as fish age | Controlling infection at the broodstock level, and use of a variably successful vaccine |
| Infection Salmon Anaemia Virus | ISA | Highly infectious, haemorrhage, fin rot can cause 100% mortality on a salmon farm | Vaccination and immediate harvest of clinical fish to prevent spread |
| *Saprolegnia spp.* | Saprolegnia | Cottony-white mycelium on fish integument, lesions, lethargy, mortality | Chemotherapeutants and good husbandry |
| *Lepeophtheirus salmonis* | Sea Lice | Gross skin lesions, lethargy and mortality with high infection level | Chemotherapeutants and site fallowing practices |

(Australian Department of Agriculture Fisheries and Forestry a, b, c 2013; Bruno 1989; Bruno 1995; Stewart 1998; World Organization for Animal Health 2013)

### 1.3 *Lepeophtheirus salmonis*

*Lepeophtheirus salmonis* is a copepod ectoparasite which colonizes primarily salmonid fish, and is commonly referred to as the sea louse or the salmon louse. *L. salmonis* has a circumpolar distribution in northern marine waters. Once *L. salmonis* have colonized a fish, they use mouth-like appendages, mandibles and mouth tube, to scrape mucus, skin and blood from the host organism sometimes resulting in extensive damage (Jonsdottir et al. 1992). The open wounds the lice create on the host reduce the ability of the fish to osmoregulate, increase susceptibility to secondary infections, and can result in reduced growth rates (Grimnes & Jakobsen, 1996). These health impacts of sea lice are of high concern in the aquaculture industry, as farming of Atlantic salmon often allows a higher than natural lice infection intensity to develop. This high infection level is a risk to the livelihood of farmers and transfer of lice from farmed fish can occur, potentially impacting wild fish populations (Reviewed by Cunningham 2006).

#### 1.3.1 *Lepeophtheirus salmonis* life cycle

The lifecycle of *L. salmonis* begins with the release of planktonic nauplii from egg strings on adult female lice (Figure 1.1). There are two nauplii stages separated by a moult, I and II. After the nauplii II stages the louse moults into a planktonic copepodid. The copepodid is the first life stage that interacts directly with the host; it is responsible for finding and attaching to a suitable host. Once a suitable host is located the louse will adhere to the surface of the fish using its second antennae and first maxilla, while it prepares for frontal filament secretion, resulting in a durable adherence to the skin. Once secreted, the frontal filament secures the louse to the host during two chalimus moults (Maran et al. 2013). Chalimus are able to feed on the skin and mucus of the fish, but are only able to feed within the range of the frontal filament. After the second chalimus moult, there is a procession of two pre-adult stages and one final adult stage.

Pre-adult females develop a large genital segment described as having a horse-shoe shape and the males have a small barrel shaped genital complex. Pre-adult and adult lice are able to move and feed freely across the surface of the fish, using their maxilla and cephalothorax to remain on the surface of the host. Pre-adult and adult lice feed primarily on the skin and mucus of fish, but consume blood meal opportunistically from the lesions that develop on the surface of the host (Johnson and Albright 1991).

### 1.3.2 Host seeking behaviour

In the life cycle of *L. salmonis* there are two phases where the lice are mobile and confronted with preferences for host selection. Copepodids must find and colonize the initial host and pre-adult/adult lice are able to move between hosts, possibly from a less suitable to a more suitable host. *L. salmonis* use both positional and chemical cues to locate a host (Mordue Luntz and Birkett 2009).

*L. salmonis* copepodids are most likely to be found in clear water, near the surface, and will adjust their position in the water column based on salinity and pressure. They perform diel vertical migration, moving to the surface of the water column during the day and to deeper water at night (Heuch, Parsons, and Boxaspen 1995). Copepodids have been shown to respond to water movement generated using a rubber fish head by displaying “attack” behaviour (Heuch, Coall and Yen 2006). Copepodids also exhibit a preference for more saline water and demonstrate a significant reduction in ability to colonize a host in sea water below 27ppt salinity compared to seawater with 36ppt salinity (Bricknell et al. 2006). In a 38K microarray study, copepodid lice exposed to low salinity water demonstrated 163 instances of differential

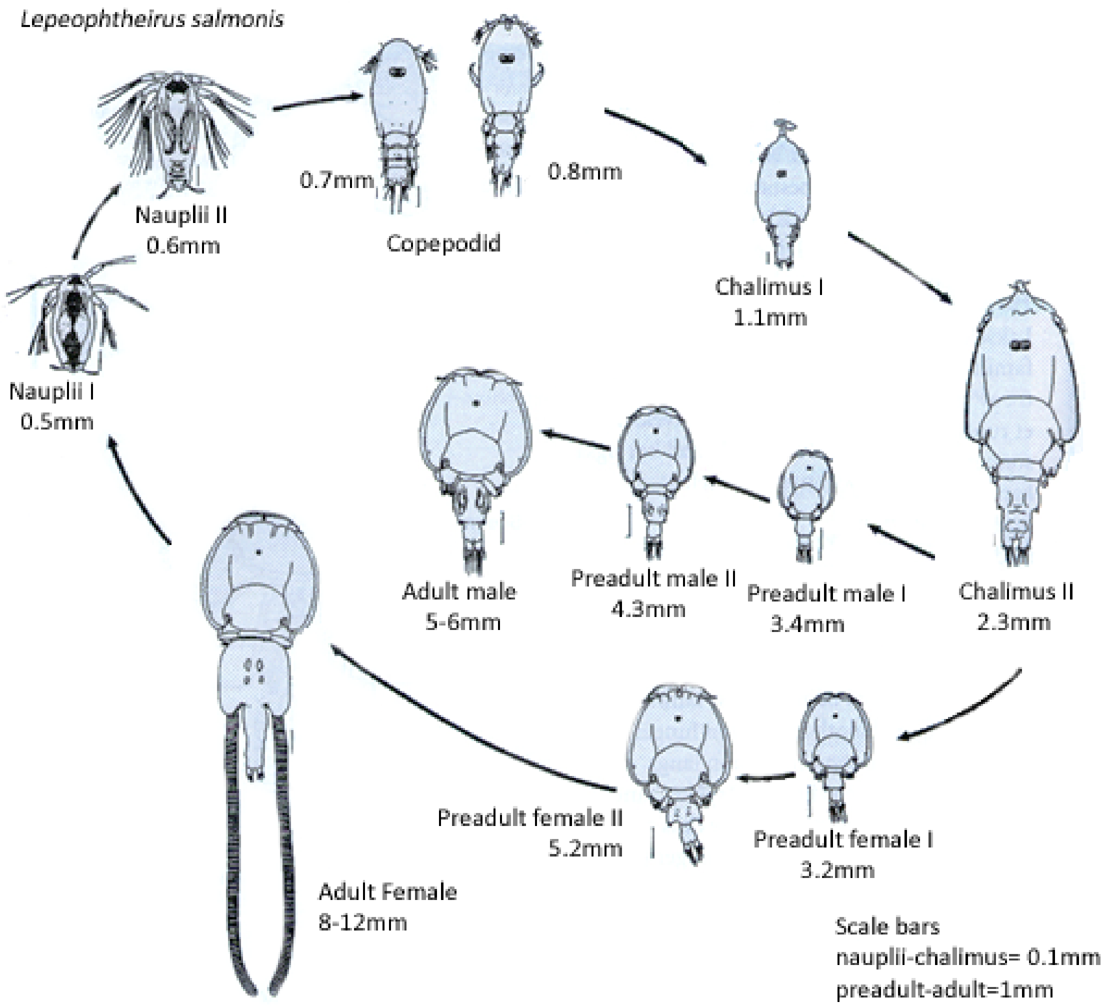


**Figure 1.1:** *Lepeophtheirus salmonis* lifecycle adapted from Schram 1993 to include updated life cycle by Maran et al. 2013

gene regulation, interpreted as a response to the abiotic stress (Sutherland et al. 2012). The ability to actively migrate within the water column helps lice to position themselves where they are most likely to encounter salmon, increasing the chance the copepodid will come into contact with a suitable host (Bricknell et al. 2006).

In addition to positional control, lice attachment is enhanced by sensory recognition of host presence and suitability. Olfactory and physical cues are used by the louse to detect the presence of a host. The louse will undergo rapid swimming behaviour to land on the surface of a potential host, where it will use chemical cues to asses host suitability (Mordue Luntz and Birkett, 2009). Figure 1.2 provides an outline of a proposed process of host identification and infection (Mordue & Birkett 2009).

**1.4 Semiochemicals**

Semiochemicals are molecules that convey information about the surrounding environment they are found in. They are released from one organism and interpreted by another organism, either of the same species or of a different species. Heuskin et al. (2011) have divided semiochemicals into two broad classes, which are further broken into small groups. Semiochemicals involved in intraspecies communications are defined as pheromones, and communicate messages involved with mate identification, aggregation, alarm, trail and host marking. Semiochemicals involved with interspecies interactions are termed allelochemicals. Allelochemicals consist of allomones, which benefit the emitter, kairomones, which benefit the receiver, and synomones, which benefit both the emitter and receiver (Heuskin et al. 2011).

Pheromones were the first semiochemical to be discovered. Bombykol was first isolated from the moth species *Bombyx mori* by Butenand et al.in 1958 (as referenced by Cork 2004). After

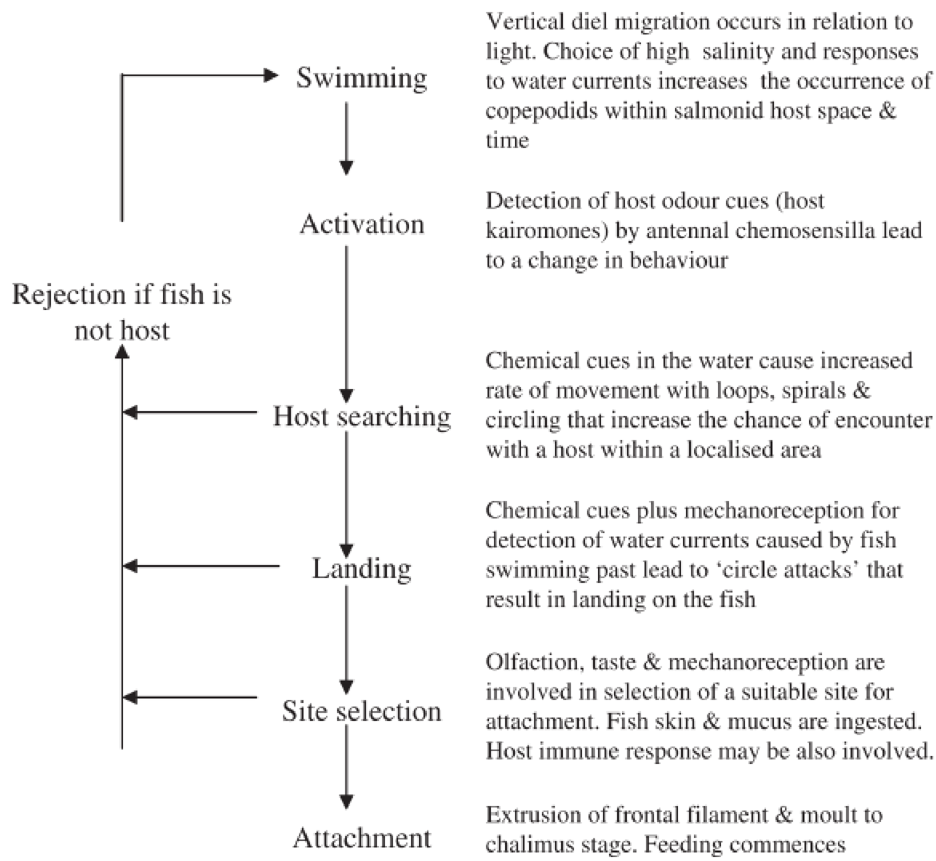


**Figure 1.2:** Proposed pattern of host seeking behaviour in copepodid *Lepeophtheirus salmonis* (Mordue Luntz and Birkett, 2009).

the discovery of bombykol the scientific community immediately recognized the potential uses of pheromones and other semiochemicals as part of an Integrated Pest Management program. There was a rush to discover as many semiochemicals as possible, although they were not immediately commercialized. Since the discovery of bombykol, several other semiochemicals have been commercially developed and have been used to help control agricultural pests (Cork 2004; Witzgall, Kirsch, and Cork 2010). It should be noted that genetic modifications of certain crops, such as cotton, has greatly reduced the need for semiochemicals use since the 1990s. However, as pests have begun to develop adaptations that allow them to feed on genetically modified plants, as exemplified by the ability of the boll weevil (*Anthonomus grandis*) to feed on genetically modified cotton, farmers are beginning to look into developing new semiochemical control methods (Cork 2004).

Use of semiochemicals has great potential to control pest species with minimal cost to the surrounding ecosystem, as their specificity reduces the potential for impact on non-target species. By removing a single compound from the complex mixture of chemicals that comprise insect pheromones it is possible to tailor pest control down to a single insect species (Cork 2004). Additional benefit of semiochemicals is that they have shown to be active in very low concentrations and are largely thought to have no adverse effects on non-target organisms (Witzgall, Kirsch, and Cork 2010).

### 1.4.1 Semiochemicals and *Lepeophtheirus salmonis*

Adult male lice and copepodids will exhibit positive taxis to various preparations of salmon conditioned water, as demonstrated using five minute Y-tube assays (Bailey et al. 2006). Several compounds have been identified as attractants to *L. salmonis*; Isophorone, 6-methyl-5-hepten-2-one, and 1-octen-3-ol (Bailey et al. 2006; Ingvarsdóttir et al. 2002; Lees et al. 2008). A rheotaxic response was not elicited when the same test was performed using a mucus sample obtained from the non-salmonid turbot, *Scophthalmus maximus* (Devine et al. 2000). To further explore the role of host specific cues, two compounds isolated from turbot conditioned water, inclusion of 2-aminoacetophenone and 4-methylquinazoline was found to reduce the attraction of copepodid *L. salmonis* to salmon conditioned to control levels (Bailey et al. 2006). This indicated the existence of specific compounds that cue host recognition of salmonids, rather than a more generalized attraction to fish.

As previously discussed, semiochemicals play an essential role in host identification and location. Farmers have been able to take advantage of semiochemicals to help protect their crops from terrestrial pests; Codlemon is used to disrupt the mating of codling moth, *Cydia pomonella*, in apple orchards, and mass trapping techniques are used to control pests that are difficult to control with traditional pesticides such as bark beetles (*Ips duplicatus*), palm weevils (*Rhynchophorus palmarum)*, and the Brinjal fruit and shoot borer (*Leucinodes orbonalis*) (Witzgall, Kirsch, and Cork 2010). A field trial using semiochemicals to control *L. salmonis* has been completed, the non-host semiochemical 2-aminoacetophenone was used to mask the attractive semiochemicals salmon emit, and reduce the number of lice per fish by 72% (Hastie et al. 2013)

### 1.5 Atlantic salmon responses to infection by *Lepeophtheirus salmonis*

As briefly mentioned previously, *L. salmonis* feed by using a mouth tube to scrape mucus and skin off of their host. The feeding of adult lice can result in the formation of large patches of eroded skin on the surface of the fish, resulting in a variety of physiological impacts. Although mobile lice (adult and pre-adult) are capable of causing easily observed clinical signs, the fish react to infection much earlier in the sea lice life cycle.

In previous infection studies of fish using copepodid lice, the fish became agitated, reacting to the parasite by jumping, flashing, and generally exhibiting more movement than is seen in fish that are not currently feeding (personal observation). Following the initial infection and moult through the chalimus life stages, the lice begin feeding on the host, although the scope of their feeding is limited by their attachment to the surface of the host through a frontal filament. The relatively small amount of damage caused by chalimus does result in some physiological changes in the fish. Mustafa et al. (2000) have observed a significant increase in the stress hormone cortisol beginning in 2 year old smolts 3 days post challenge (dpc) with copepodid lice, while 1 year old smolts did not exhibit a change in cortisol level until 7dpc. In the same study, increases in plasma glucose levels were significantly increased in the 2 and 1 year old smolts at 7 and 14dpc, respectively. Changes in macrophage functions were observed by 21dpc, when the majority of lice had moulted to pre-adults (Mustafa et al. 2000). Other work has demonstrated that Atlantic salmon head kidney macrophages exhibit a significant suppression of both respiratory burst activity and phagocytic activity 14 and 21 dpc. In addition to the change in macrophage function there were transient increases in the alkaline phosphatase and lysozyme biochemistry of the salmon mucus (Fast et al. 2002). These changes in fish immune molecules and defence mechanisms may be occurring due to an immunosuppressive secretion by *L. salmonis* or as a result of damage to skin tissue from physical damage caused during attachment and feeding.

Unlike chalimus, appearance of mobile lice on Atlantic salmon results in an increase of cortisol and tumour-necrosis factor-like gene expression, and a transient increase in the major histocompatibility class I and II genes (Fast et al. 2006). A microarray study by Skugor et al. (2008) looked at differential gene regulation in salmon skin tissue, using skin samples noted to be either intact or damaged, during *L. salmonis* infection of Atlantic salmon. There were several trends in observed differential regulation following the progression of lice to the adult phase, notably an increase in the anti-inflammatory genes interleukin 1 and tumor growth factor-β. There was an increase in arginase I suggesting the initiation of a Th2 immune response, a response pathway indicating challenge by a multicellular parasite. Additionally, several genes associated with calcium signalling and muscle contraction were upregulated at the chalimus phases and subsequently down regulated at the pre-adult phases, several genes associated with wounded tissue were upregulated and several genes associated with wound healing were differentially regulated in a way that suggested impaired wound healing (Skugor et al. 2008).

### 1.6 *Lepeophtheirus salmonis* modulation and impact on Atlantic salmon during infection

All host dependent stages of *L. salmonis* are known to secrete low molecular weight proteins while feeding (Fast et al. 2003). Louse secretions are known to contain both trypsins and the lipid compound prostaglandin E2 ($PGE_2$), among other as of yet unidentified compounds (Wagner, Fast, and Johnson 2008). While the role of secretions from *L. salmonis* have only recently begun to be elucidated, previous research suggests they have an important role in host modulation and/or feeding (Firth, Johnson, and Ross 2000). Although not well defined in sea lice, the role of secreted molecules as a means of increasing ease of feeding by parasites has been well documented in terrestrial pests. $PGE_2$ is found in the saliva of several tick species, and has a role in suppressing interleukin-2 expression in the host (Aljamali et al. 2002). It has also been shown that $PGE_2$ has anti-hemostatic, anti-inflammatory and vasodilatory effects (Aljamali et al. 2002; Riveiro et al. 1985, 1992). In *L. salmonis,* trypsins are considered to be general digestive peptidases, and are thought to be secreted onto the surface of a host to aid in feeding. Other low molecular weight proteins can be found in the mucus of *L. salmonis* infected fish, while absent in the mucus of uninfected fish (Kvamme et al. 2004).

Once moulted to the mobile pre-adult and adult life stages, the potential impact of *L. salmonis* feeding becomes much higher than at the copepodid and chalimus stages. The chalimus stages, which range from approximately 1.2 to 2.8mm in length, probably cannot consume the same amount of mucus and skin as the mobile stages, which range from 2.9 to 5.4 mm in pre-adult and adult male lice and from 3.7 to 10.0mm in pre-adult I and adult female lice in length (Johnson and Albright 1991). An experimental challenge to Atlantic salmon with copepodid lice was able to demonstrate that *L. salmonis* infected Atlantic salmon tended to have decreased growth and body condition factor (Grimnes and Jakobsen 1996). During this study, no mechanical damage was observed on the fish prior to the development of pre-adult lice. On the third day following the first observation of pre-adult lice, 31% of fish had lesions and on the fifth day of pre-adult infection up to 73% of fish had lesions (Grimnes and Jakobsen 1996). This is biologically relevant, as mucus and skin are the first defence mechanisms fish have against pathogen invasion. Mobile lice often aggregate on the head, between the dorsal and caudal fins and in the perianal regions of the fish, which can result in the formation of gross lesions. Smaller holes and fissures were observed on the surface of the fish using scanning electronic microscopy (Jarworski and Wolm, 1992 and Jonsdottir et al. 1992). Lice have also been noted to insert the front of their cephalothorax underneath scales, likely to circumvent the protection offered to the fish by their scales (Jonsdottir et al. 1992).

### 1.7 *Lepeophtheirus salmonis* control methods

A variety of management practices and chemotherapeutant applications have helped to manage the impacts of *L. salmonis* infection in marine aquaculture. Unfortunately, environmental concerns and development of drug resistance have made management a continually evolving practice.

### 1.7.1 Good management practices

Canada uses a variety of measures to help reduce disease occurrence in its aquaculture industry, including a requirement for all stock to be certified disease free before entering the marine environment, use of biosecurity measures, and fallowing practices (Department of Fisheries and Oceans Canada 2012a). In Atlantic Canada most salmon farms are located in the Bay of Fundy, famous for its extreme tides. The movement of water can result in water displacement of 146km$^2$ in 6.2 hours, with rises and falls over 40ft, the highest tides in the world (Desplanque and Mossman 2001). This large volume of water exchange, while useful for maintaining water quality on farms, poses a challenge to limiting spread of disease.

Like many other countries, Canada has implemented a number of best management practices. One vital tool is the use of Aquaculture Bay Management Areas (ABMA). Farms in each ABMA are strongly encouraged to work together to synchronize their farming practices, including husbandry, harvesting, year class stocking, fish health and fallowing procedures. Within each ABMA this agreement can be changed or negotiated at the beginning of each production cycle, and procedures to ensure and enforce compliance are included in each agreement (Department of Agriculture, Fisheries, and Oceans Canada, accessed August 19, 2013). By ensuring that each farm in the ABMA is using proper husbandry techniques, it increases the overall health of the region by decreasing the incidence of disease, and fallowing helps to break the lifecycle of any parasites or other disease causing agents (Atlantic Canada Fish Farmers Association 2012b). While the lifecycle of *L. salmonis* can be difficult to interrupt, as many salmonid species can act as a reservoir, fallowing does allow the number of copepodid and adult lice in the ABMA to be drastically reduced before the beginning of the next production cycle (Stewart 1998).

In 2002 a survey was issued to workers on 83 aquaculture sites in the Bay of Fundy to garner information on farming practices in the area in relation to ISA and sea lice infection. It was found that over 90% of sites had regular visits from a veterinarian, and the remaining 10% had visits from a veterinarian on a less regular basis. Most sites had frequent monitoring of lice levels on the farm, 48.2% of farms performing at least biweekly counts in the summer months, with other sites performing counts monthly or as needed. Counts were performed to help determine if lice infection had reached a critical level where treatment was required, and to be able to prepare for high adult lice numbers based on the presence of the chalimus stages (Westcott, Hammell, and Burka 2004). Today farms are required to submit their lice counts and any pesticide applications to a shared database, which helps to determine yearly trends in infection and monitor for the development of drug resistance in louse populations. Counts must be submitted either weekly or monthly depending on the water temperature (Department of Fisheries and Oceans 2011a). This information can help farms and BMAs manage lice levels, by allowing them to identify factors that may lead to increased or decreased success in management practices.

### 1.7.2 Chemotherapeutant administration

Unfortunately, in mariculture *L. salmonis* cannot be controlled solely through the use of improved husbandry techniques. The use of chemotherapeutants has become vital in the control of this parasite in open sea cage culture systems. Many chemotherapeutants used to control *L. salmonis* infection have been adapted from land based uses in agriculture and animal husbandry, however these treatments tend to affect a broad array of organisms, making them poorly adapted for use in aquatic environments (Burridge et al. 2010). Chemotherapeutants in aquaculture are typically delivered in one of two ways, via an external exposure to the drug in a bath treatment or exposure to a drug by ingestion via a medicated feed.

Chemotherapeutants delivered as a bath treatment are considered to be pesticides, while those delivered as in-feed are termed drugs (Department of Fisheries and Oceans 2013b). There are pros and cons to both of these treatment methods. There are three common methods of delivering a bath treatment: skirt, tarp and well boat exposures. The most simplistic of the three, the skirt treatment, has largely fallen out of favour due to the uncontained nature of the treatment. Skirts are made of weighted tarpaulin that surrounds or partially surrounds a sea cage to 8-10m in depth. Once the skirt is in place, pesticide is added to the sea cage. Since skirts can be partially open, and do not cover the bottom of the sea cage, there can be water exchange between the surrounding environment and the water in the cage surrounded by the skirt. The movement of water into and out of the skirt system dilutes the drug introduced into the cage, lessening the treatment dose, sometimes to a lower concentration than is effective. Skirts are still used when inclement weather or strong tides render a tarpaulin treatment too dangerous to apply (Corner et al. 2008). Tarpaulin treatments are similar to skirt treatments, in that they are executed in the sea cage. Unlike the skirt treatment, the tarpaulin completely surrounds the sea cage. The closed bottom of the tarpaulin system prevents the diffusion of drug out of the cage (Department of Fisheries and Oceans Canada 2013b). With both the skirt and tarpaulin systems, it can be difficult to determine the amount of water enclosed by the tarpaulin, as tides and human error can affect the positioning of the tarpaulin, leading to large variations in the volume of enclosed water. This variation in treatment volume can lead to over or under application of a pesticide, with reported variations in expected water volume ranging from 0.46-1.66 fold of the predicted water volume (Treasurer, Grant, and Davis 2000). This change in variation could lead to under or over application of a pesticide, potentially leading to mortality events should the pesticide concentration be too high, or contribute to the development of resistance toward the pesticide during low or sub-optimal application.

Well boat bath treatments are able to counteract many of the problems associated with skirt and tarpaulin treatments. In a well boat treatment, fish are pumped out of their cage into a well aboard a ship. This well is filled with a known volume of water, and the appropriate amount of pesticide is added. The fish remain in the well for the duration of treatment and are subsequently pumped back into their sea cage (Department of Fisheries and Oceans Canada 2013b). Well boat treatments can be executed in a wider range of weather conditions than both skirt or tarpaulin treatments, however, up to four separate treatments may be required to treat all of the fish within a single sea cage. Well boat treatments can use up to 50% less pesticide due to the concentration of fish within the relatively small volume of water in the well (Department of Fisheries and Oceans Canada 2013b). Economically, the cost of crew, equipment and barge for skirt treatments is approximately $7, 200 daily, while the cost for well boat treatment approaches $12, 000, both methods of treatment allowing farmers to treat 4-6 cages daily. The reduced amount of drug required for well boat treatments offsets the increased daily cost, requiring an average of $800 of pesticide per cage, while tarpaulin treatments require approximately $2,400 of pesticide per cage. Well boat treatments also offer a more effective treatment, as pesticides doses can be delivered more accurately (Atlantic Canada Fish Farmers Association 2012a). Following an initial pilot study in 2010 to determine the effectiveness of well boat treatments, three boats were purchased for use in New Brunswick, Canada at an estimated cost over 38 million dollars, and have been used in other provinces and in the United States (Lynn Hutchin, Affiliation of DAAF, NB, Pers Com). A major advantage of all types of bath treatments is ensured exposure of all fish to the pesticide, given that the treatment is properly executed. A major disadvantage to bath treatments is the large release of parasiticides to the environment, which have the potential to significantly impact non-target species (Haya, Burridge and Davies 2005).

In-feed treatments are delivered to fish as they feed, allowing the drug to be incorporated into the fish feed. This makes the application of treatment a much simpler undertaking; simply replacing the standard feed with a medicated feed requires minimal additional effort (Grant 2002). The most common in-feed treatment is emamectin benzoate, which has been shown to provide significant protection against sea lice infection for 50-80 days post cessation of treatment (Skilbrei et al. 2008; Stone et al. 2000). This is substantially longer than the protection provided through bath treatments, which need to be applied more frequently to maintain protection (Davies and Rodger 2000). The downside of many in-feed treatments is over or under treatment of individual fish, competition for food may result in some fish not consuming any food during treatment while other fish may consume much more than the therapeutic dose. Additionally, fish may find the medicated feed unpalatable or due to stress of infection cease feeding resulting in treatment failure (Grant 2002). An ecological benefit to in feed treatments is the relatively small release of drug into the environment, as most of it is consumed by the fish (Haya and Davies 2005).

### 1.7.3 *Lepeophtheirus salmonis* chemotherapeutants

Internationally, there have been a number of different chemical classes developed for use during sea lice infection: Avermectins, chitin synthesis inhibitors, organophosphates, pyrethroids and hydrogen peroxide (Atlantic Canada Fish Farmers Association 2012b). Due to concerns about therapeutic margins, environmental impacts, and development of resistance not all drug classes are still used. Table 1.2 outlines availability and use of several compounds in Canada. Only five chemotherapeutants are currently available for use in Canada, and the

**Table 1.2:** Common *Lepeophtheirus salmonis* chemotherapeutants, their availability, and application.

| Chemical Class | Compound | Commercial Name | Canadian release type and dates | Effective against | Delivery | Method of action |
|---|---|---|---|---|---|---|
| Avermectins | Emamectin benzoate | SLICE® | Currently registered | Host dependent lice | In-feed | Causes the release of GABA from nerve endings and increases binding of GABA on receptors. Leads to inhibition of neurotransmission |
| | Ivermectin | Ivomec® | Off-label use | | | |
| Chitin Inhibitors | Teflubenzuron | Calicide® | Currently registered | Moulting lice | In-feed | Inhibits the proper formation of chitin |
| | Diflubenzuron | Lepsidon® | NCA | | | |
| Organophosphates | Azamethiphos | Salmosan® | Registered in 1990s Emergency registration 2010-2013 | Mobile lice | Bath | Inhibits acetylcholinesterase activity |
| | Dichlorvos | Aquaguard® | NCA | | | |
| Pyrethroids | Cypermethrin | Excis® | NCA | Host dependent lice | Bath | Interference with sodium channels in axon membranes |
| | Deltamethrin | Alphamax® | Emergency registration 2008-2010 | | | |
| | High-*cis* cypermethrin | Betamax® | NCA | | | |
| Hydrogen Peroxide | $H_2O_2$ | Salartect® | NCA | Mobile lice | Bath | $H_2O_2$ is thought to cause mechanical paralysis through the liberation of oxygen in the gut and hemolymph. |
| | $H_2O_2$ | Paramove® | Emergency registration since 2010 | | | |

NCA: Not Currently Available; Host dependent lice: Chalimus I – adult; Mobile lice: Pre-adult I – adult; Moulting lice: Lice transitioning between stages
(Atlantic Canada Fish Farmers Association 2012b; Bright and Dionne 2005; Burridge et al. 2010; Davies and Rodger 2000; Grant 2002)

limitations of each treatment can make it difficult to manage lice infection levels. For instance Paramove® can only be safely administered in water temperatures below 12°C, and due to administration in a well boat, bath treatments often cannot be executed on all sea cages within a site during a single day. This leaves reservoirs of untreated fish that can re-infect the treated fish. Administration of both bath and in-feed treatments can require a mandatory withdrawal period before being processed for human consumption. (Atlantic Canada Fish Farmers Association 2012a, 2012b; Bright and Dionne 2005). Also worth noting is that the majority of the treatments available for use in Canada have been released under emergency registration, and may have that registration revoked should the treatment be found to have severe health or environmental impacts (Atlantic Canada Fish Farmers Association 2012b).

### 1.7.4 Development of drug resistance in *Lepeophtheirus salmonis* populations

Currently, one of the most important factors in the ability to control *L. salmonis* infection on salmon farms is the limited number of chemotherapeutants available. Furthermore, lice have begun to develop drug resistance to the available chemotherapeutants. Five chemotherapeutants may initially appear to be a substantial number of products to rotate in an effort to prevent the development of drug resistance, but to put it in perspective there are 325 pesticide products registered to treat fleas in dogs in Canada, and this count excludes drug products (Pesticide label search: fleas% & dogs%, http://pr-rp.hc-sc.gc.ca/ls-re/index-eng.php , November 22 2013)

In the early 1990s fish farmers in Scotland began reporting that increased treatment frequency of the organophosphate dichlorvos (Aquaguard®) was required to manage *L. salmonis* infection. *In vitro* work was able to demonstrate varying tolerance to dichlorvos across lice populations, indicating the development of resistance (Jones, Sommerville, and Wootten 1992). Due to the development of resistance, small therapeutic margin, and potential environmental impacts, dichlorvos has fallen out of favour as a sea lice treatment in Scotland (Rae 2002). As dichlorvos use declined, use of a second organophosphate, azamethiphos (Salmosan®), increased. It was found that this compound could result in nearly 100% removal of lice on some farms, but had lower success on others, which some groups attributed to multidrug resistance in certain lice populations (Roth et al. 1996). More recently, organophosphates are no longer commonly used to treat *L. salmonis* due to resistance (Burridge et al. 2010).

Pyrethroids experienced a similar progression of resistance development as occurred with organophosphates, with reports of treatment failures in the early 2000s and bioassay confirmation of resistant populations (Sevatdal and Horsberg 2003). Investigation into the mechanism of resistance to pyrethroids began by seeking similar resistance mechanisms as found in terrestrial arthropod pests. It has been demonstrated that by exposing *L. salmonis* to piperonyl butoxide, an inhibitor of cytochrome p450 monooxygenases, increased sensitivity to both cypermethrin and deltamethrin can be elicited (Sevatdal et al. 2005). A second potential method of resistance development is genetic point mutations. A mutation has been identified within the para-type sodium channel in the nerve membrane of pyrethroid resistant *L. salmonis* populations, and this mutation is not found in susceptible populations. The highly conserved region this mutation was found in suggests that rather than a non-functional polymorphism, this mutation confers some selective advantage (Fallang et al. 2005).

Following the failure of organophosphates as a lice management tool, avermectin use became a popular control method. Ivermectin was briefly used in Scotland, but due to the high toxicity and small therapeutic margin to both fish and other organisms in the ecosystem, its use was discontinued (Davies and Rodger 2000). A second avermectin, emamectin benzoate (Slice®), was developed to replace ivermectin. Slice® was capable of drastically reducing lice numbers while having a lower toxicity and wider margin of error than ivermectin. Recently there are many accounts of treatment failures due to resistance development against SLICE® in Europe, Chile and Atlantic Canada (Lees et al. 2008; Whyte et al. 2011, Igboeli et al. 2012). Some of the mechanisms for lice resistance to Slice® have begun to be elucidated. P-glycoprotein (P-gp) has been identified as a ATP-dependent drug efflux pump that enhances *L. salmonis* resistance to emamectin benzoate, similar to resistance to ivermectin in mammals and other arthropods (Prichard and Roulet 2007). Use of verapamil to inhibit P-gp function increases the toxicity of Slice® to sea lice, and lice that survive high Slice® exposure show increased P-gp expression (Igboeli et al. 2012).

### 1.8 Research goals

Due to the development of resistance, high cost, and need for frequent application of chemotherapeutants, developing alternative methods of sea lice control has become a necessity.

The current project has two aims, the first to help elucidate the interactions that occur between *L. salmonis* and Atlantic salmon during host switching and colonization. This may identify signals and trends in gene expression that lead to successful colonization or rejection of a host. The second objective is designed to investigate the effectiveness of an anti-attachment factor as a tool for interrupting successful host colonization using an *in vitro* model.

Objectives:

1) Elucidate mechanisms associated with host switching in adult *L. salmonis*
    a. Use a cohabitation of *L. salmonis* infected and uninfected salmon to determine host selection preference.
        i. Ho: Lice will have no host preference
        ii. Hi: Lice will prefer either initially infected of initially uninfected fish
    b. Use gene expression analysis to determine if any differential gene expression occurs in *L. salmonis* during the host switching process.
        i. Ho: There will be no difference in gene expression between groups
        ii. Hi: There will be differences in gene expression between groups
    c. Use gene expression analysis to determine if any differential gene expression occurs in the Atlantic salmon during the host switching process.
        i. Ho: There will be no differences in gene expression between initially infected and initially uninfected Atlantic salmon
        ii. Hi: There will be differences in gene expression between initially infected and initially uninfected Atlantic salmon
    d. Use correlative analysis to determine the relationship between host and parasite interactions.
        i. Ho: There will be no correlation in gene expression between the host and parasite
        ii. Hi: There will be correlations in gene expression between the host and parasite
    e. Use correlative analysis to determine if the relationship between host and parasite interaction increase on fish with relatively low or high lice loads.

i. Ho: Infection intensity will have no effect on the correlations between host and parasite gene expression

ii. Hi: Infection intensity will have an effect on the correlations between host and parasite gene expression

2) Determine if exposure to an anti-attachment factor alters *L. salmonis* behaviour, survival, or gene expression in an *in vitro* model.

   a. Monitor survival of copepodid and adult lice during exposure to an anti-attachment factor at increasing dosages.

      i. Ho: Allyl isothiocyanate will have no effect on lice survival

      ii. Hi: Allyl isothiocyanate will have a negative effect on lice survival

   b. Use a plate bioassay to determine if differential gene expression occurs in copepodid or adult lice exposed to an anti-attachment factor.

      i. Ho: Allyl isothiocyanate will have no effect on lice gene expression

      ii. Hi: Allyl isothiocyanate will have a dose dependent effect on lice gene expression

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# CHAPTER 2

## COMPARISON OF HOST SELECTION AND GENE EXPRESSION OF ADULT *LEPEOPHTHEIRUS SALMONIS* AND *SALMO SALAR* DURING A COHABITATION OF INITIALLY INFECTED AND UNINFECTED FISH

This chapter has been prepared as the following manuscript to be submitted for publication:

**Comparison of host selection and gene expression of adult *Lepeophtheirus salmonis* and *Salmo salar* during a cohabitation of initially infected and uninfected fish**

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## 2.1 Abstract

*Lepeophtheirus salmonis* is a common parasite of salmonid fish and has a significant economic impact on Atlantic salmon (*Salmo salar*) fish farms. Over time *L. salmonis* has developed resistance to a number of chemotherapeutants, making the discovery of new treatments important to maintain a profitable farming industry. Determining processes in both *L. salmonis* and Atlantic salmon important to host selection and colonization may provide new targets for treatment development. During a two week cohabitation of *L. salmonis* infected and uninfected Atlantic salmon, we were able to collect information on the ability of *L. salmonis* to switch hosts, and preference for infected or uninfected fish. Whole *L. salmonis* and Atlantic salmon tissues were collected at 2 and 14 days post cohabitation to determine if differential gene expression was occurring during this process. At 2 days post cohabitation there was no significant difference in the number of male lice on the initially infected and uninfected fish. Eight *L. salmonis* genes putatively associated with various facets of lice survival (CYP18 A1-like, cytochrome p450 Isoform 1-like protein, glycene receptor α-2-like, leukocyte receptor cluster member 9-like, nicotinic acetylcholine receptor subunit-like, tissue plasminogen activator precursor-like, peroxinectin-like, and Trypsin-1) were analysed in both adult male and female lice, as well as five genes indicating immune status in Atlantic salmon. Comparisons were made to look for differential gene regulation as well as correlation between expression of *L. salmonis* genes and Atlantic salmon genes. Only MMP9 expression in salmon spleen was differentially regulated during the study period, however, correlations between the expression of several louse and salmon genes were found. Notably, the expression of a peroxinectin-like gene in male and female *L. salmonis* was correlated with the expression of IL-1, IL-12, IgT and matrix metalloproteinase 9 intermittently in salmon.

## 2.2 Introduction

*Lepeophtheirus salmonis* is a species of parasitic crustacean commonly referred to as sea lice. This species of lice has a circumpolar distribution, infecting fish in the marine waters of the Northern Hemisphere. *Lepeophtheirus salmonis* is primarily a parasite of salmonids, and is particularly pathogenic to Atlantic salmon (*Salmo salar*). The life cycle of *L. salmonis* has recently been revised and consists of eight stages; two nauplii and one copepodid free swimming stages, two chalimus stages which are completely host dependent and sessile on the surface of the host, and finally two preadult moults and an adult stage which freely move across the surface of the fish (Maran et al. 2013, Nilsen Pers. Com.).

Shortly after commercial fish farming began in Europe, salmon farmers began to notice large numbers of *L. salmonis* colonizing their fish. By the early 1970s, salmon farmers were already beginning to use chemotherapeutants to remove lice (Brandal and Egidius 1973). Management of *L. salmonis* infection on farmed Atlantic salmon is imperative to the aquaculture industry, as sea lice can cause significant damage to salmon as they feed. As the lice feed on skin and mucus, lesions can occur and will result in difficulty maintaining osmoregulatory balance, decreased growth rates, secondary infections and decreased quality of life for the fish (Grimnes and Jakobsen 1996, Bowers et al. 2000, Mustafa et al. 2000). Total impact of sea lice infestation of Atlantic salmon on the aquaculture industry has a cost of nearly $500USD million annually, due to reduced value of final product and use of parasiticides (Costello 2009).

In the past two decades salmon farmers have noted the development of resistance in *L. salmonis* populations to commonly used parasiticides (Lees et al. 2008). This has increased the associated costs caused by the parasite by necessitating the use of higher doses of chemotherapeutants or requiring more frequent treatments to reduce infections to acceptable levels. Development of resistance has necessitated research into new control strategies. Ingvarsdóttir et al. (2002) showed that *L. salmonis* host seeking behaviour can be activated by both Atlantic salmon conditioned water and *L. salmonis* conditioned water. The importance of chemical cues in *L. salmonis* host settlement initiated investigation into attractant chemicals as well as those that mask the cues used to identify an appropriate host. By understanding the mechanisms and pathways behind successful host recognition and settlement initiation, we may be able to interfere with this process and identify novel ways of controlling infection.

The following study was designed to characterize gene regulation during the identification and colonization of Atlantic salmon by the sea louse species *L. salmonis* and to identify corresponding changes in immune responsiveness of Atlantic salmon. Cohabitation of Atlantic salmon initially infected and initially uninfected with adult *L. salmonis* allowed us to determine the rate at which the parasites switch hosts, observe any differentially regulated genes, and make correlations between the expression of these genes in both the host and the parasite during these events.

**2.3 Study Objectives**

Elucidate mechanisms associated with host switching in adult *L. salmonis*

a. Use a cohabitation of *L. salmonis* infected and uninfected salmon to determine host selection preference.

    i. $H_i$: Lice will prefer either initially infected of initially uninfected fish

b. Use gene expression analysis to determine if any differential gene expression occurs in *L. salmonis* during the host switching process.

    i. $H_i$: There will be differences in gene expression between groups

c. Use gene expression analysis to determine if any differential gene expression occurs in the Atlantic salmon during the host switching process.
   i. $H_i$: There will be differences in gene expression between initially infected and initially uninfected Atlantic salmon
d. Use correlative analysis to determine the relationship between host and parasite interactions.
   i. $H_i$: There will be correlations in gene expression between the host and parasite

## 2.4 Materials and methods

### 2.4.1 Ethics statement

Fish in this experiment were treated as required by the Canadian Council on Animal Care, the specific protocol used was approved by University of Prince Edward Island Animal Care Committee (Protocol Number: 10-014). Sampled fish were handled minimally and rapidly euthanized using an overdose of Tricaine methanesulfonate (TMS) (Syndel Laboratories, Qualicum Beach, BC) ($250mg_{TMS}/1L_{Tank\ water}$) to minimize stress.

### 2.4.2 Cohabitation study design

Atlantic salmon were housed at the aquatics facilities of the Atlantic Veterinary College in 330L tanks with a closed recirculating artificial salt water system. Mean weight of all fish was 246.1±6.8g SEM and mean fish length was 27.6 ± 0.3 cm. Salinity was maintained at 34ppt (±2ppt) and temperature at 11$^{o}$C (±1$^{o}$C); ammonia, dissolved oxygen, nitrate and nitrite levels were monitored weekly. Photoperiod was an 8h:16h light:dark cycle. During the study fish were fed 1% initial body weight of a 3.5mm commercial salmon feed (Corey Nutrition Feed, Fredericton, NB, http://www.coreyaqua.ca) split between two daily feedings.

Cohabitation entailed adding 14 *L. salmonis* infected Atlantic salmon into a tank containing 14 uninfected Atlantic salmon, repeated in a duplicate tank. Average lice load of initially infected fish was 4.0 ±0.54 lice/fish. Infected Atlantic salmon had been infected with laboratory reared *L. salmonis* copepodids 72 days prior to the beginning of cohabitation, and individual lice numbers on each infected fish was recorded as it was transferred into the study tanks. The *L. salmonis* hatching and the initial infection procedure followed that described by Covello et al. (2012). Due to the duration of time between initial infection of fish and commencement of cohabitation, all lice in this study had moulted to the adult life stage. Uninfected fish were labelled with PIT tags one month prior to the beginning of the study to allow differentiation between initially infected and uninfected fish.

At 2 and 14 days post cohabitation (dpc) 7 initially uninfected and 7 initially infected fish were collected from each tank using a dip net, the first 7 from each group that were captured were euthanized and sampled. Weight and length was recorded for each fish, and PIT tag number was recorded for fish that were tagged. Louse gender, stage, and total number were also recorded for each fish, and lice from each individual fish were collected into a 1.5mL microcentrifuge tube and stored on dry ice for the duration of sampling. Approximately 300mg of spleen and a section of skin approximately 2cmx4cm were collected from a standardized site dorsal and posterior to the pectoral fin from each sampled salmon. Collected salmon tissues were stored on dry ice for the duration of sampling. On completion of sampling all samples were transferred and stored at -80$^{o}$C. Lice found on initially uninfected fish were considered to have switched hosts, whereas those found on and initially infected host were considered to have not switched hosts.

### 2.4.3 RNA extraction

RNA was extracted from adult male and adult female lice whole body homogenates using a Tri-Reagent extraction method (Chomczynski 1993). Lice were divided into four treatment groups defined by whether they had switched host during the cohabitation trial, consisting of: 1) lice that remained on an Initial Host at 2dpc, 2) lice that remained on the Initial Host at 14dpc, 3) lice that moved to a New Host at 2dpc, and 4) lice that moved to a New Host at 14dpc. No female lice were observed to have switched hosts, and therefore only the Initial Hosts at 2 and 14dpc treatment groups exist for analysis of female lice. Ten lice were selected from each group to undergo RNA extraction, with an effort to select lice representing each duplicate tank, and minimizing use of multiple lice from the same fish. RNA was also extracted from the spleen and skin samples of the salmon from which the lice were collected. RNA from salmon tissues was extracted from 100-200mg of each respective tissue. RNA samples were resuspended in molecular grade water and tested using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE) to confirm purity and concentration.

Lice RNA generally had low 260/280 values, the lowest being 1.62 and an average ratio of ~1.80. Analysis of RNA quality using a BioRad Experion (Mississauga, ON), showed generally acceptable RNA Quality Indicator (RQI) values on a subset of samples, the lowest value being 4.7 and the highest being 9.8. Although some of these samples had an RQI <6, there did not appear to be any problems during downstream processing of the lower quality samples, and they were included in analysis. All salmon tissue RNA had 260/280 ratios greater than 1.85 and RQI values >7.0, with one exception of 4.2.

After RNA extraction, 1µg of each RNA sample was treated with a DNase (TURBO DNA-*free*™ kit, Ambion, Carlsbad, CA) to prevent genomic DNA from interfering with downstream processing. Treatment followed the manufacturer's protocol. Following DNase treatement samples were reverse transcribed (GoScript™ Reverse Transcription System, Promega, Madison, WI) using the manufacturer's protocol.

### 2.4.4 *Lepeophtheirus salmonis* genes

A list of eight genes associated with different facets of *L. salmonis* survival, feeding, suppression of Atlantic salmon immune response, metabolism and neurotransmission were selected for gene expression analysis. Selected genes had been identified as being differentially regulated in a 38K oligonucleotide array examining the effects of emamectin benzoate exposure on *L. salmonis*. Putative gene functions were assigned by performing a BLASTx search in both the Swiss-Prot and Non-redundant protein sequence databases (blast.ncbi.nlm.nih.gov/Blast.cgi) with the sequences of interes,t and using results with a Nr-Evalue and Swissprot-Evalue ≤ $1xE^{-10}$ (Table 2.1).

Primers were designed from *L. salmonis* sequences using Primer 3 software (simgene.com/primer3) and are shown in Table 2.2. Amplified polymerase chain reaction (PCR) sequences were run on 2% agarose gels to confirm presence of a single amplicon; amplicons were confirmed via sequencing (Macrogen USA, Rockville, MD).

### 2.4.5 Atlantic salmon genes

Five salmon genes of interest associated with salmon immune responses to *L. salmonis* were selected for qPCR analysis; Interleukin (IL)-1β, IL-8, IL-12, Immunoglobulin T (IgT), and Matrix Metalloproteinase 9 (MMP9). These genes and primer sets were previously shown to be appropriate immunological markers for response to sea lice in Atlantic salmon (Purcell et al. 2013).

**Table 2.1:** Brief description of *Lepeophtheirus salmonis* putative gene functions

| **Gene** | **Function** |
|---|---|
| CYP18 A1-like | Ecdysteroid involved in moult and metamorphosis (Guittard et al. 2011) |
| Trypsin-1 | Serine protease involved in feeding, digests mucus and skin (Johnson et al. 2002) |
| Tissue plasminogen activator precursor-like | Degrades blood plasma proteins preventing clotting (Prevot et al. 2006) |
| Cytochrome p450 isoform 1-like Protein | Drug metabolism of aromatic hydrocarbons (Baldwin et al. 2009) |
| Peroxinectin-like | Louse immune system, encapsulation of invaders (Jiravanichpaisal et al. 2006) |
| Leukocyte receptor cluster member 9-like | Immune response (Zucchetti et al. 2009, Johansson 1999) |
| Glycene receptor α-2 Like | Mediates inhibitory neurotransmission (Lynch 2009) |
| Nicotinic acetylcholine receptor subunit-like | Excitatory neurotransmitter receptor (Le Novère and Changeux 1995) |

**Table 2.2:** *Lepeophtheirus salmonis* primer sets

| **Gene Name** (Swissprot-ID) | **Forward/ Reverse** | **Sequence** |
|---|---|---|
| **CYP18 A1-like** (sp\|Q95078\|CP18A_DROME) | Forward | TGGGAGGTGAAACCGTCGTAGT |
| | Reverse | CCCCCAGAAGCTGGGATAACTCTGT |
| **Tryspin-1** (sp\|P00765\|TRYP_ASTFL) | Forward | TGGTCGCAACTGCTCTTGCA |
| | Reverse | GGCTCTGCCTCTTCACCACCG |
| **Tissue plasminogen activator precursor-like** (sp\|P11214\|TPA_MOUSE) | Forward | AGGGAAATGCCATGGTGTGCAACT |
| | Reverse | TGACACCATCATTACACGACCTCGT |
| **Peroxinectin-like** (sp\|Q9VEG6\|PERC_DROME) | Forward | TGGGCTTTGGCCGCTCCAAA |
| | Reverse | GGCTGTGTCCGAATCGAAAGGCA |
| **Leukocyte receptor cluster member 9-like** (sp\|Q96B70\|LENG9_HUMAN) | Forward | AGGTATACGGGAAGGCACAGACCT |
| | Reverse | TGGCCAAAGGTACCCAGTCCT |
| **Cytochrome p450 isoform 1-like** (sp\|Q9VG82\|CP9F2_DROME) | Forward | TTGGGGTGGAAGCAGGCTGC |
| | Reverse | ACGCAAAAACCAATGCTTGTCTCCA |
| **Glycene receptor, α-1 like** (sp\|P19019\|GBRB3_CHICK) | Forward | ACGACGCTTCACGTGTGGAGT |
| | Reverse | AGAGGCCCGGAAAGTTGTTGAGT |
| **Nicotinic acetylcholine receptor α6 subunit-like** (Q9VG82) | Forward | CTCTGCCGCACATCCACCCC |
| | Reverse | TGGTGGAGGCGGAGGCTGAT |

Primer sequences used to determine if differential gene expression in *L. salmonis* occurred during a cohabitation study of infected and uninfected Atlantic salmon. Putative functions were assigned by BLASTx searching full *L.* salmonis sequences in both the Swiss-Prot and nr databases and using a consensus to determine function.

### 2.4.6 Gene quantification

Relative gene expression was determined using quantitative real time PCR (qPCR). Quantitative PCR reactions were performed in duplicate with 96 well plates, using GoTaq® qPCR Master Mix (Promega, Madison, WI ), on a Mastercycler® ep Realplex model 2S thermocycler (Eppendorf, Mississauga, ON). Replicate samples with a difference in threshold cycle (cT) greater than 0.55 were re-run in duplicate to a maximum of three times, and if still outside the variance limit were excluded from analysis. Reaction conditions were optimized by generating a standard curve with appropriate efficiencies (0.9-1.1) and $R^2$ values (>0.95). Genes of interest were amplified using a 3-step protocol, while reference genes were amplified using a 2-step protocol (Tables 2.3 and 2.4).

Three reference genes were used for normalization of *L. salmonis* gene expression; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), eukaryotic translation elongation factor 1α (EF-1α) and structural ribosomal protein S20 (RPS20). Three reference genes were also used to normalize Atlantic salmon gene expression; EF-1α, elongation initiation factor, and RPS20. Reference gene stability was determined using GeNorm$^{Plus}$ software (M value≤1.0, CV≤0.5) (Biogazelle, Zwijnaarde, Belgium); a fourth reference gene (18S rRNA gene) was excluded from *L. salmonis* analysis as it was identified as being unstable.

Samples were normalized using qBASE software (Biogazelle, Zwijnaarde, Belgium) by comparing expression of the genes of interest to the expression of the reference genes to generate a Mean Normalized Referenced Quantity (MNRQ) (Hellemans et al. 2007).

**Table 2.3:** *Lepeophtheirus salmonis* quantitative PCR reaction parameters

| **Gene** | **Annealing Temperature (°C)** | **Standard Curve Efficiencies** | **Protocol** |
|---|---|---|---|
| CYP18 A1-like | 60 | 1.02 | 3 Step |
| Trypsin-1 | 65 | 0.99 | 3 Step |
| Tissue plasminogen activator precursor-like | 60 | 1.07 | 3 Step |
| Cytochrome p450 isoform-1 like Protein | 65 | 1.07 | 3 Step |
| Peroxinectin-like | 60 | 0.99 | 3 Step |
| Leukocyte receptor cluster member 9-like | 65 | 1.00 | 3 Step |
| Glycene receptor α-2 like | 65 | 0.99 | 3 Step |
| Nicotinic acetylcholine receptor subunit-like | 65 | 1.06 | 3 Step |
| Ribosomal Protein S20 | 61.0 | 0.92 | 2 Step |
| Glyceraldehyde 3-phosphate dehydrogenase | 65.1 | 0.97 | 2 Step |
| Elongation Initiation Factor 1 | 65.1 | 0.91 | 2 Step |

Quantitative PCR protocols used to amplify reference genes and genes of interest in *Lepeophtheirus salmons*. Three step protocols consist of: One cycle of 95°C for 10 minutes, forty cycles of 95°C for 15 seconds, $T_{Annealing}$°C for 15 seconds and 72°C for 15 seconds, and one melt curve. Two step protocol consisted of: One cycle of 95°C for 10 minutes, forty cycles of 95°C for 15 seconds, $T_{Annealing}$°C for 30 seconds and one melt curve.

**Table 2.4:** Atlantic salmon quantitative PCR reaction parameters

| Gene | Annealing Temperature (°C) | Standard Curve Efficiencies | Protocol |
|---|---|---|---|
| Interleukin 1β | 55 | 0.91 | 3 Step |
| Interleukin 12 | 55 | 0.93-1.04 | 3 Step |
| Immunoglobulin T | 55 | 0.93-0.95 | 3 Step |
| Matrix Metalloproteinase 9 | 55 | 0.90-0.91 | 3 Step |
| Interleukin 8 | 55 | 0.91-0.93 | 3 Step |
| Elongation Factor 1 | 55 | 0.91-0.93 | 3 Step |
| Elongation Factor | 55 | 1-1.06 | 3 Step |
| Ribosomal Protein S20 | 55 | 0.93-0.95 | 3 Step |

Quantitative PCR protocols used to amplify reference genes and genes of interest in Atlantic salmon. Three step protocols consist of: One cycle of 95°C for 10 minutes, forty cycles of 95°C for 15 seconds, $T_{Annealing}$°C for 15 seconds and 72°C for 15 seconds, and one melt curve.

### 2.4.7 Statistical analysis

Statistical analysis of MNRQ values were completed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). For each gene, within each treatment, samples with an MNRQ greater than two standard deviations from the group mean were removed from the respective data set as outliers. The MNRQ values for both *L. salmonis* and Atlantic salmon were checked to ensure that within each treatment group the data set was normally distributed using either the D'Agostino & Pearson test or the Kolmogorov-Smirnov test for groups with smaller sample numbers. A one-way ANOVA was performed using a Tukey's Test ($p<0.05$) to determine if there was a significant difference between normally distributed treatment groups. If within a gene, one of the treatment groups was not normally distributed or the treatment groups were found to have significantly different standard deviations using a Brown-Forsythe test, the non-parametric Mann-Whitney test was used to determine if differential gene regulation was occurring between treatment groups for that gene. Both parametric and non-parametric tests were used to achieve the highest statistical power within each gene.

A second series of analyses were completed to determine if there was a correlation between the expression of salmon immune markers in the skin and spleen tissue of the salmon host, and also if there were correlations between *L. salmonis* gene expression and Atlantic salmon gene expression. Gene expression of each sampled *L. salmonis* was compared to the expression of the immune markers in the tissues of the Atlantic salmon from which the louse was collected using a Pearson correlation. Comparisons were made across the entire duration of the study, as well as a comparison of gene expression within each treatment group.

Finally, Pearson correlations were used to determine if salmon with a high louse infection had stronger correlations in expression between lice and salmon gene expression than fish with low numbers of lice. The initial categorization of fish as initially infected or uninfected hosts is not used for this analysis. Due to the limited number of lice in this study, fish with 0-3 lice were considered to have a low number of lice and fish with 4-14 lice were considered to have a high infection. Five fish and twenty lice were used in the high group, and seven fish and twelve lice were used in the low group. Comparisons were made in the same manner as the Pearson correlations used to elucidate correlation across the entire study.

**2.5 Results**

**2.5.1 Host selection**

Combined lice counts from both tanks indicated that male lice would transfer between hosts, while no female lice switched host (Table 2.5). After initiation of cohabitation lice were able to switch between hosts, at 2dpc there was no significant difference between the number of male lice on the Initially Infected and Initially Uninfected treatment groups. At 14dpc there was also no significant change in the number male lice on fish from both treatment groups (Table 2.5). Of the 111 lice that entered the study, 102 were recovered during sampling. All male lice were

recovered (72/72) while nine female lice (30/39) were lost during the two week study period. This indicates that adult male lice are able to choose and move to a new host relatively successfully, but adult female lice lack either the desire or ability to transfer between hosts.

**2.5.2 *Lepeophtheirus salmonis* gene expression**

A one-way ANOVA with Tukey's test determined there were no significant changes in *L. salmonis* gene expression between treatment groups or over time during the two week cohabitation study in either male or female lice (Figure 2.1 and 2.2). A Bartlett's test identified a statistically significant decrease in variation of Trypsin 1 expression in male lice that switched hosts.

**Table 2.5:** Movement of *Lepeophtheirus salmonis* during a 2 week cohabitation study

| | | Initially Infected Fish | | | Initially Uninfected Fish | | |
|---|---|---|---|---|---|---|---|
| **Tank** | **Days post cohabitation** | **Number of Fish** | **Number of Male Lice** | **Number of Female Lice** | **Number of Fish** | **Number of Male Lice** | **Number of Female Lice** |
| 1 | 0 | 14 | 40 | 19 | 14 | 0 | 0 |
| 4 | 0 | 14 | 32 | 20 | 14 | 0 | 0 |
| 1 | 2 | 7 | 9 | 4 | 7 | 8 | 0 |
| 4 | 2 | 7 | 16 | 16 | 7 | 3 | 0 |
| 1 | 14 | 7 | 11 | 9 | 7 | 9 | 0 |
| 4 | 14 | 7 | 8 | 1 | 7 | 8 | 0 |

Movement of *L. salmonis* (lice) between initially infected and initially uninfected Atlantic salmon hosts. Day 0 post cohabitation (dpc) lice counts were done immediately before initiation of cohabitation. Number of fish indicates the number of fish lethally sampled to collect and count *L. salmonis*. There was no significant difference in the number of male lice/fish between groups ($p<0.05$). No female *L. salmonis* were observed to have switched hosts.

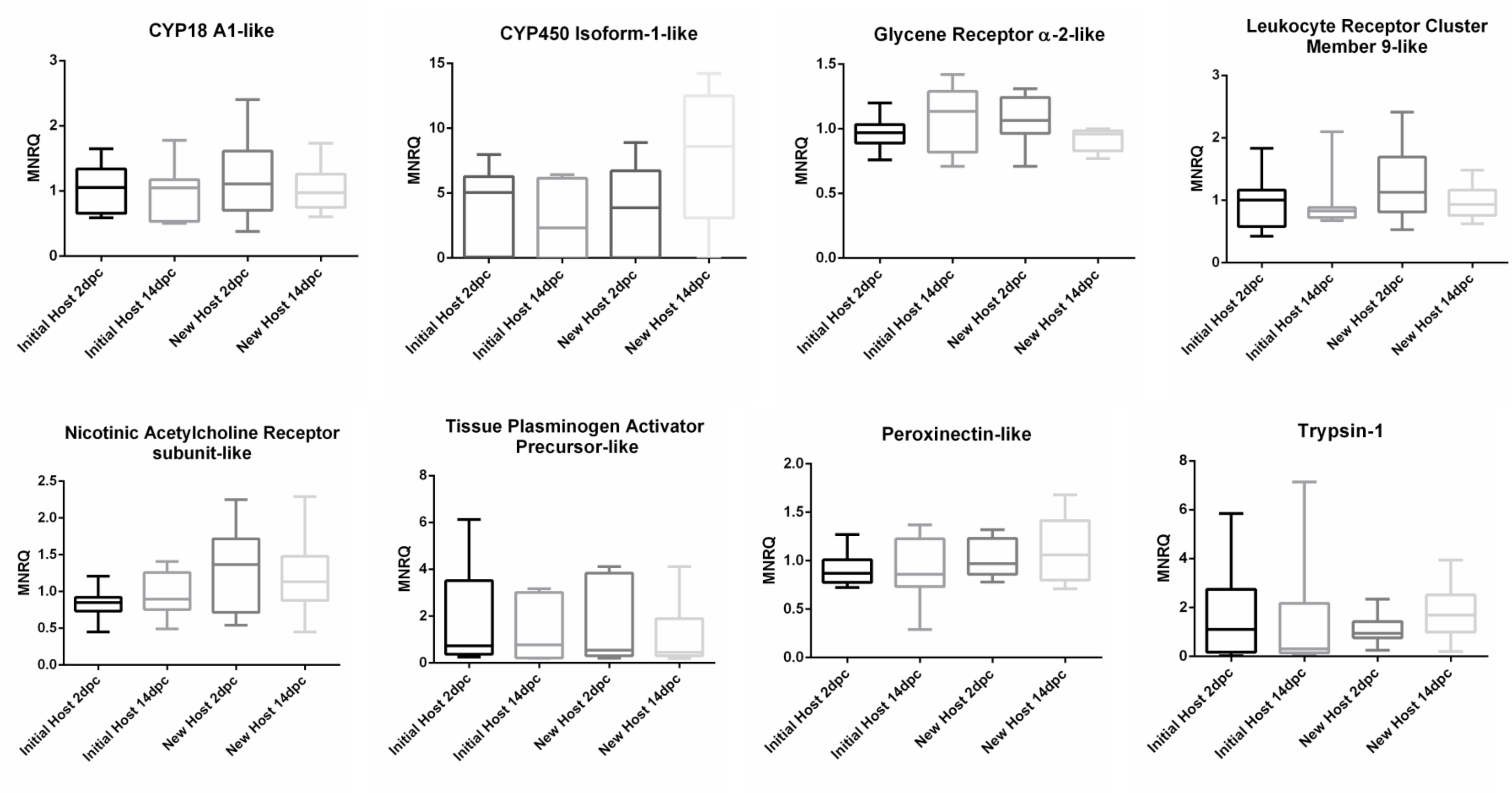


**Figure 2.1** Mean normalized relative quantity (MNRQ) of 8 adult male *Lepeophtheirus salmonis* gene expression over a two week cohabitation study. *L. salmonis* were grouped based on movement between initially infected (Initial Host) and initially uninfected (New Host) Atlantic salmon (*Salmo salar*) (n=10/group) at 2 and 14 days post cohabitation (dpc). The median is shown by the centre line. Boxes contain the 25$^{th}$-75$^{th}$ percentiles and whiskers show the range of data.

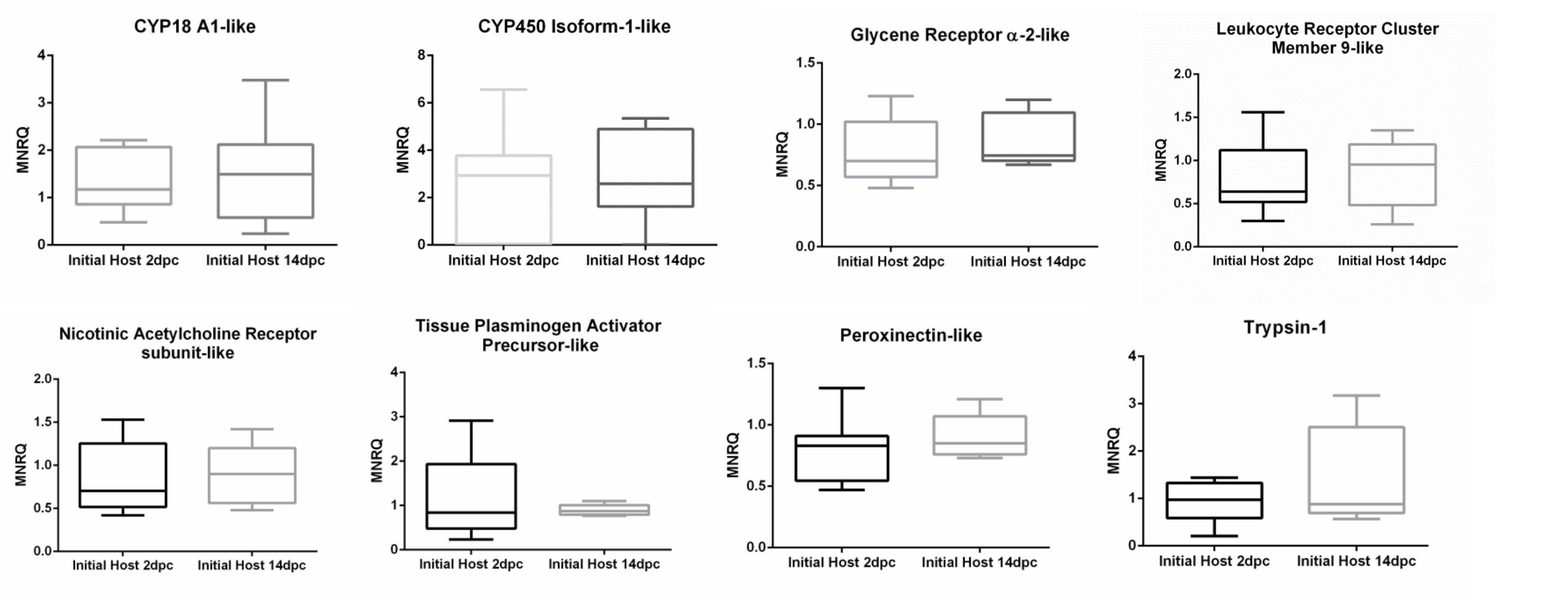


**Figure 2.2** Mean normalized relative quantity (MNRQ) of 8 adult female *Lepeophtheirus salmonis* gene expression over a two week cohabitation study. *L. salmonis* were grouped based on movement between initially infected (Initial Host) and initially uninfected (New Host) Atlantic salmon (*Salmo salar)* (n=10/group) at 2 and 14 days post cohabitation (dpc). The median is shown by the centre line. Boxes contain the 25$^{th}$-75$^{th}$ percentiles and whiskers show the range of data.

### 2.5.3 Atlantic salmon gene expression

Two sets of analysis were used to determine if differential gene regulation was occurring in Atlantic salmon, the first being fish associated with infection by male lice, and the second being with fish. In the salmon there was only one instance of a significant change in gene expression in either salmon skin and spleen involving higher expression of MMP9 in the spleen of fish associated with infection by adult male lice in the Initial Host group at 2dpc as compared to those on New Hosts at 2dpc (Fig 2.3A, 2.3B and 2.4).

### 2.5.4 Salmon skin-spleen interactions

Pearson correlations were completed to determine if any instances of significant correlations between the expression of immune markers in skin and spleen tissues had developed during the study (Table 2.6). This analysis determined that there was a positive correlation between IL-1 and IL-8 in spleen tissue with IL-12 expression in skin tissue. A full set of data is provided in Appendix A.

### 2.5.5 Host- parasite interactions

As there were no major trends within salmon or lice gene expression, analysis of individual interactions were performed. Due to tagging of fish and enumeration of lice on individual hosts, we were able to compare lice gene expression to the individual salmon from which it was collected. Using a Pearson correlation, relationships between the host and parasite began to be elucidated.

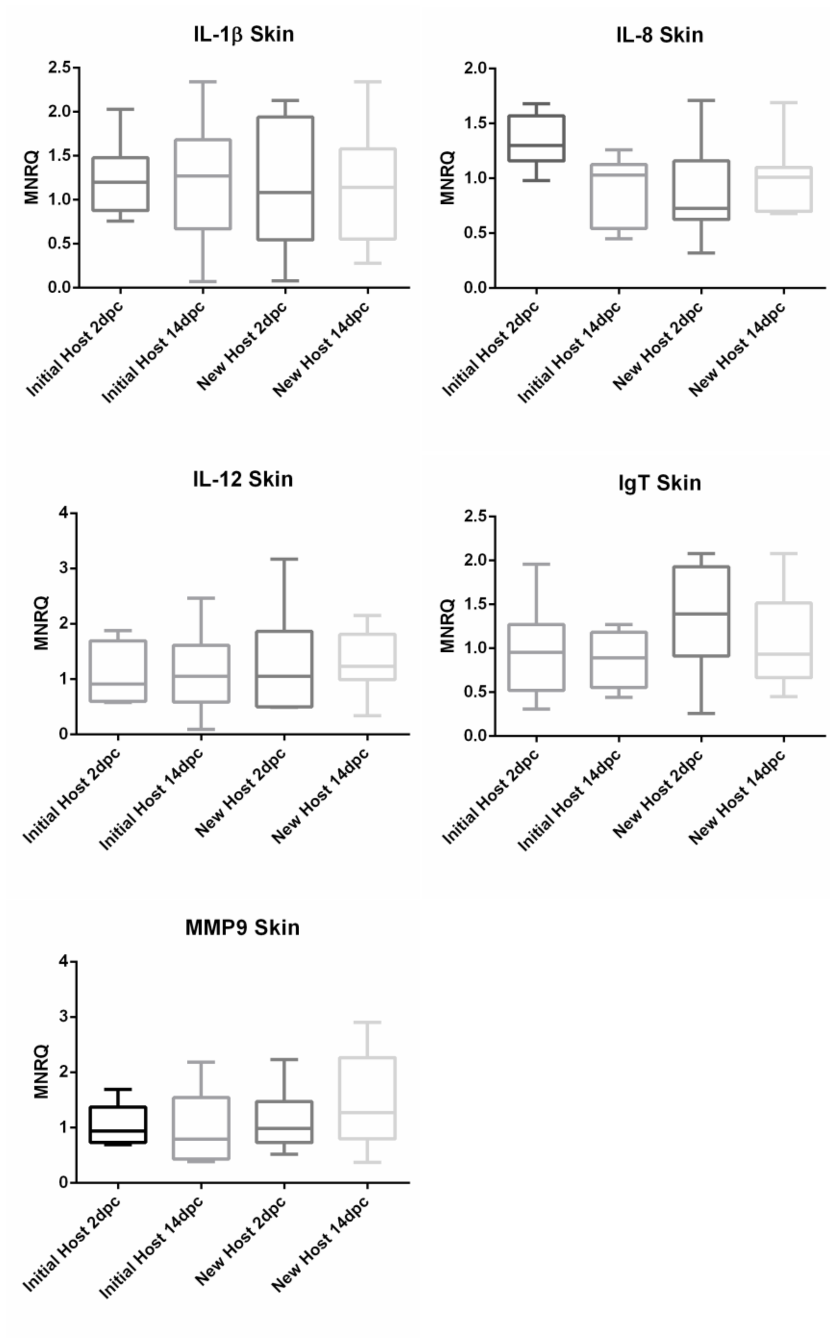


**Figure 2.3A** Mean normalized relative quantity (MNRQ) of the expression of five Atlantic salmon immune genes occurring during a two week cohabitation of *Lepeophtheirus salmonis* initially infected and initially uninfected fish. The median is shown by the centre line, boxes contain the $25^{th}$-$75^{th}$ percentiles and whiskers show the range of data.

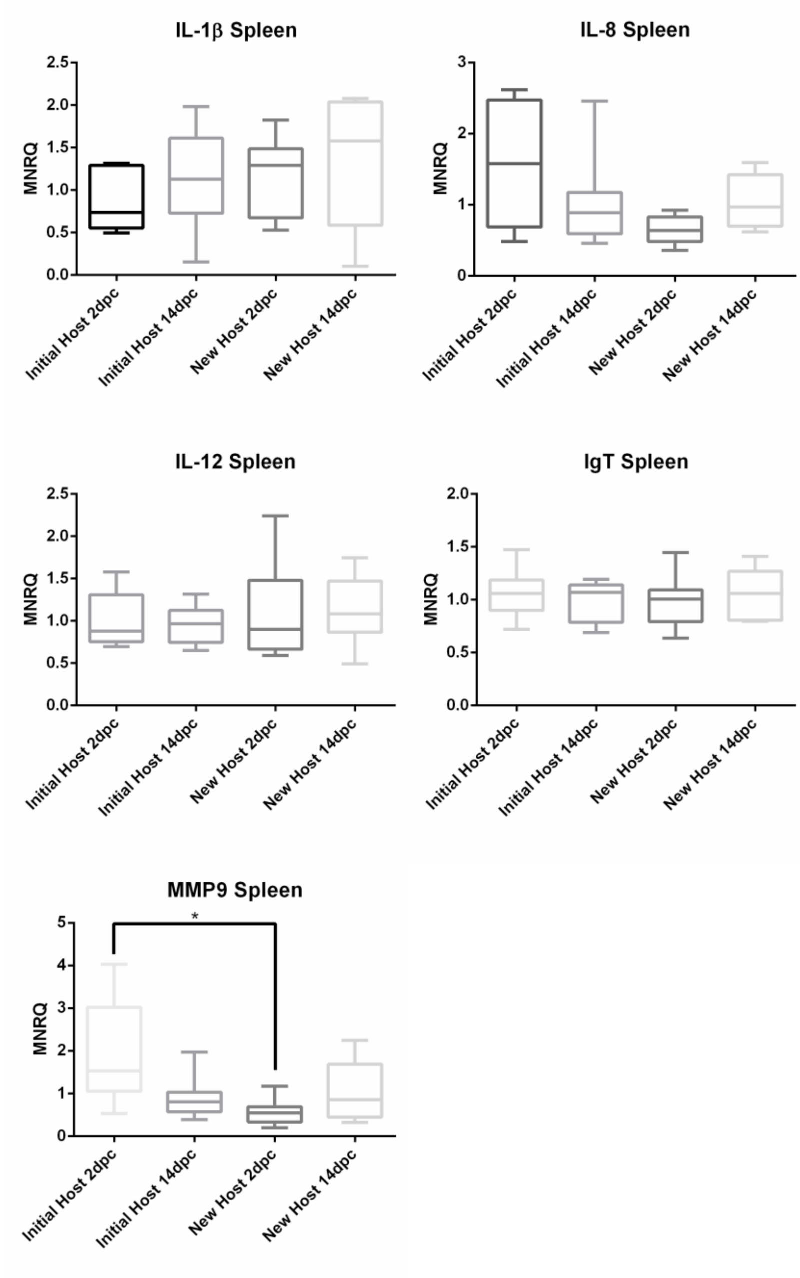


**Figure 2.3B** Mean normalized relative quantity (MNRQ) of the expression of five Atlantic salmon immune genes occurring during a two week cohabitation of *Lepeophtheirus salmonis* infected and uninfected fish. The median is shown by the centre line, boxes contain the 25th-75th percentiles and whiskers show the range of data Significant difference between groups is indicated by: "*".

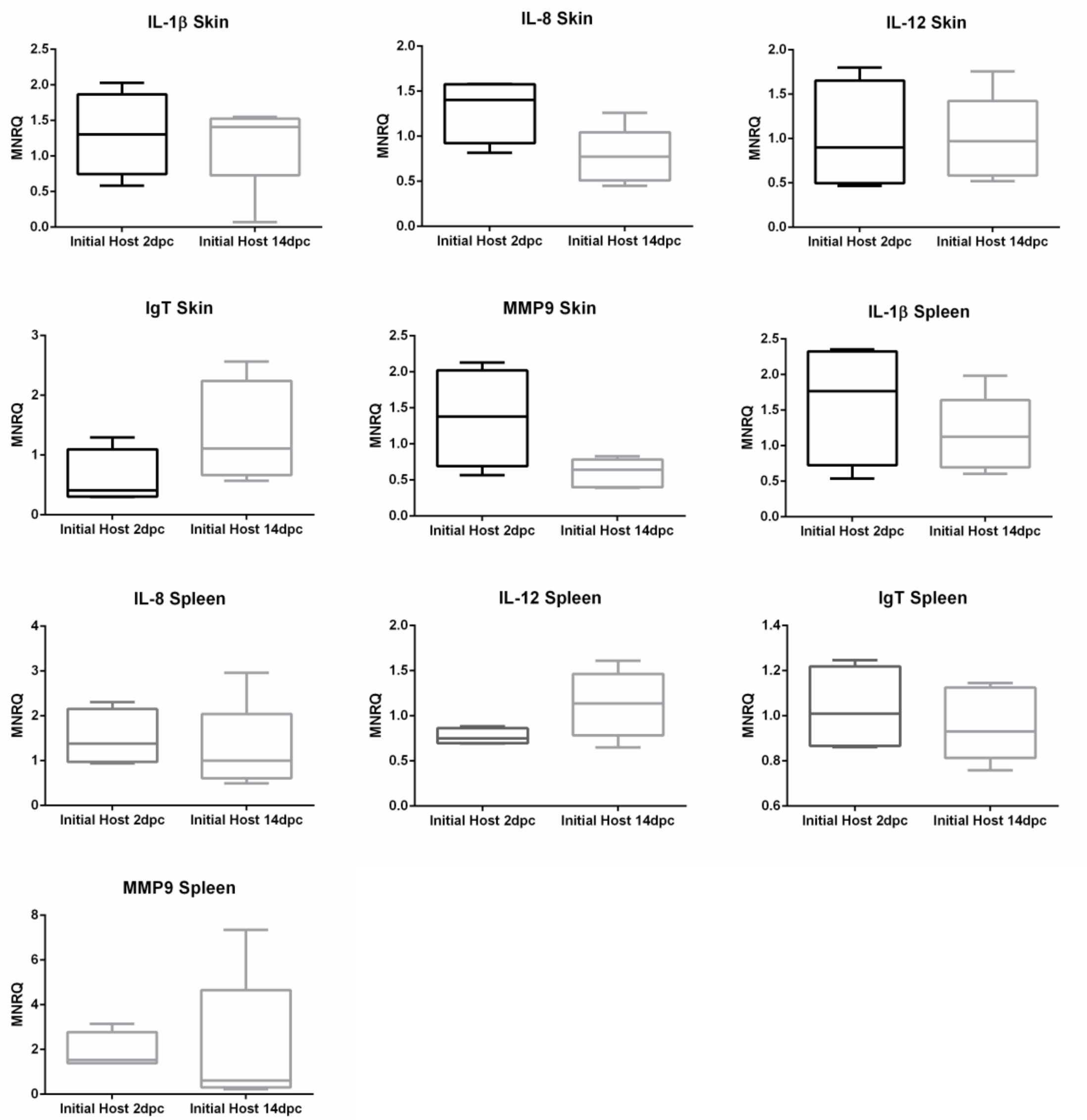


**Figure 2.4** Mean normalized relative quantity (MNRQ) of the expression of five Atlantic salmon immune genes occurring during a two week cohabitation of *Lepeophtheirus salmonis* infected and uninfected fish. The median is shown by the centre line, boxes contain the 25th-75th percentiles and whiskers show the range of data.

**Table 2.6:** Pearson correlations between expression of Atlantic salmon immune markers in salmon skin and spleen tissues

| | Initial Host 2dcp | | | | | Initial Host 14dpc | | | | | New Host 2dcp | | | | | New Host 14dpc | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Skin** | IL-1 | IL-8 | IL-12 | IgT | MMP9 | IL-1 | IL-8 | IL-12 | IgT | MMP9 | IL-1 | IL-8 | IL-12 | IgT | MMP9 | IL-1 | IL-8 | IL-12 | IgT | MMP9 |
| **Spleen** | | | | | | | | | | | | | | | | | | | | |
| IL-1 | | | | | | | | | | | | | + | | | + | | | | |
| IL-8 | | | | | | | | | | + | | | + | | | | | | | |
| IL-12 | - | | | | | | | | | | | | | | - | | | | | |
| IgT | | | | | | | | | | | | | | | | | | | | |
| MMP9 | | | | - | | | | | | | | | | | | | | | | |

Summation of the Pearson correlations between gene expression in Atlantic salmon skin and spleen tissues. Interleukin (IL)-1, IL-8, IL-12, immunoglobulin T (IgT) and matrix metalloproteinase 9 (MMP9) were analyzed. Positive (+) and negative (-) correlations with a p-value less than 0.05 are shown.

There were several instances where there was a single correlation between male louse gene expression and the gene expression in Atlantic salmon skin tissue within each of the four treatment groups. There were multiple instances of correlation of male louse expression of the peroxinectin-like gene to Atlantic salmon skin gene expression in the Initial Host groups (Table 2.7), as well as correlations between Atlantic salmon skin gene expression of IL-8 to louse expression of CYP450 I1-like (Pearson C:-0.844, P-value: 0.017) and nicotinic acetylcholine receptor subunit-like (Pearson C: 0.717, P-value: 0.045) in the Initial Host 2dpc group associated with colonization by female lice. Some fish were colonized by both male and female and were therefore included in both sets of analyses.

The correlation between peroxinectin-like gene expression in *L. salmonis* and expression of Atlantic salmon genes in skin tissue were present again in the female lice Initial Host treatment groups (Table 2.8). Unlike the male lice, there were also correlations between peroxinectin-like expression and gene expression in Atlantic salmon spleen (Table 2.8 A&B). Additionally, Atlantic salmon IL-12 gene expression was positively correlated with expression of several female *L. salmonis* genes, peroxinectin-like in the Initial Host at 2dpc treatment group (Pearson C: 0.676, P-value: 0.0455), and with both the glycene receptor α2-like (Pearson C: 0.760, P-value: 0.028) and Trypsin-1 (Pearson C: 0.828, P-value: 0.005) expression in Initial Hosts at 14dpc. In male lice, correlations between the expression of peroxinectin-like gene in lice and markers of salmon immune status tended to increase in strength as time progressed (Table 2.7). The entirety of the time-based correlative data can be viewed in Appendix B.

**Table 2.7:** Correlation between the response of adult male *Lepeophtheirus salmonis* Peroxinectin-like gene expression and the response of Atlantic salmon skin tissue

| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Pearson C.** | **P-value** | **Pearson C.** | **P-value** | **Pearson C.** | **P-value** | **Pearson C.** | **P-value** | **Pearson C.** | **P-value** |
| **Initial Host (2dpc)** | -0.025 | 0.944 | -0.498 | 0.173 | 0.049 | 0.894 | **0.641** | **0.046** | -0.399 | 0.254 |
| **Initial Host (14dpc)** | **-0.759** | **0.011** | -0.657 | 0.054 | -0.533 | 0.140 | **0.726** | **0.042** | -0.436 | 0.208 |
| **New Host (2dpc)** | -0.358 | 0.344 | -0.321 | 0.400 | -0.084 | 0.844 | -0.453 | 0.221 | -0.219 | 0.572 |
| **New Host (14dpc)** | 0.603 | 0.086 | -0.097 | 0.820 | -0.582 | 0.078 | -0.349 | 0.357 | -0.284 | 0.427 |

Pearson correlation (Pearson C.) of the mean normalized relative quantity (MNRQ) of adult male *Lepeophtheirus salmonis* Peroxinectin-like gene expression and the MNRQ of five markers of Atlantic salmon immune response in skin tissue. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p≤0.05 are significantly different than 0.

**Table 2.8 A & B:** Correlation between the response of adult female *Lepeophtheirus salmonis* peroxinectin-like gene expression and the response of Atlantic salmon skin and spleen tissues

| **A** | **IL-1** | | **IL-8** | | **IL-12** | | **IgT** | | **MMP9** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Pearson C.** | **P-value** | **Pearson C.** | **P-value** | **Pearson C.** | **P-value** | **Pearson C.** | **P-value** | **Pearson C.** | **P-value** |
| **Skin: Initial Host (2dpc)** | 0.434 | 0.243 | 0.630 | 0.069 | **0.676** | **0.046** | **0.681** | **0.043** | **0.667** | **0.050** |
| **Skin: Initial Host (14dpc)** | 0.087 | 0.824 | -0.093 | 0.813 | 0.476 | 0.195 | 0.569 | 0.141 | 0.398 | 0.289 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult female *Lepeophtheirus salmonis* peroxinectin-like gene expression and the MNRQ of five markers of Atlantic salmon immune response in skin tissue. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

| **B** | **IL-1** | | **IL-8** | | **IL-12** | | **IgT** | | **MMP9** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Pearson C.** | **P-value** | **Pearson C.** | **P-value** | **Pearson C.** | **P-value** | **Pearson C.** | **P-value** | **Pearson C.** | **P-value** |
| **Spleen: Initial Host (2dpc)** | **0.687** | **0.041** | 0.219 | 0.571 | -0.621 | 0.075 | **0.668** | **0.049** | 0.657 | 0.054 |
| **Spleen: Initial Host (14dpc)** | -0.006 | 0.988 | -0.125 | 0.749 | 0.210 | 0.588 | 0.120 | 0.759 | -0.430 | 0.248 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult female *Lepeophtheirus salmonis* peroxinectin-like gene expression and the MNRQ of five markers of Atlantic salmon immune response in spleen tissue. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

There were changes in significant correlations between *L. salmonis* and salmon gene expression when differentiating between fish with low and high infection levels. In correlations examining male lice and salmon skin there were three instances of negative correlation between fish and louse in the high infection group and one instance of positive correlation. In the low infection group there was one instance of negative correlation and seven instances of positive correlation. In the comparisons between male lice and salmon spleen tissues there were three instances of positive correlation in the high infection group and one instance of positive correlation in the low infection group. In female lice and salmon skin comparisons there was one instance of negative correlation in the high infection group and two instances of positive correlation, in the low infection group there were six instances of positive correlation. Comparisons of female lice and salmon spleen revealed three instances of negative correlations and one instance of positive correlation in the high infection group, and three instances of positive correlation in the low infection group. This data is summarized in Table 2.9, and the entirety of the infection intensity correlative data can be viewed in Appendix C.

## 2.6 Discussion

### 2.6.1 Host selection

It was found that the male lice reached an equal distribution across all hosts, although female lice appear to have remained on the same fish for the duration of the study. Previous research has shown that adult male and preadult II female *L. salmonis* are able to switch hosts in both field and laboratory studies (Ritchie 1997). Another study using a related sea lice species, *Caligus elongatus,* found that male and female adult lice without a host could find and attach to a number of fish species over a period of several hours (Øines et al. 2006). The lack of movement of adult female *L. salmonis* observed in this study compared to the movement of

**Table 2.9:** Summation of instances of correlation between *Lepeophtheirus salmonis* and Atlantic salmon based on infection intensity

| Lice Load | Correlations in Host Skin | Correlations in Host Spleen |
|---|---|---|
| Less or equal to 3 | +13 and -1 | +4 and -0 |
| Greater or equal to 4 | +3 and -4 | +4 and -3 |

Summation of the number of instances of significant positive and negative Pearson correlations between both male and female lice and the expression of several markers of Atlantic salmon immune status.

preadult II *L. salmonis* and adult *C. elongatus* in the two previous experiments may be attributed to morphological and energical demands associated with reproduction. The larger genital segment of adult female *L. salmonis* would be cumbersome and could potentially reduce swimming efficiency. As these lice were already situated on an appropriate host, energy allocation to host seeking could potentially impair reproduction and be disadvantageous to the genetic success of the parasite.

Redistribution of male lice may be a survival strategy where pressure on each individual louse is minimized. The equal distribution of lice would result in reduced competition for food, space on the host and access to potential virgin female lice. It has been shown that *L. salmonis* exhibit positive taxis towards both salmon and lice conditioned water in Y-tube experiments (Ingvarsdottir et al. 2002). It is possible that male *L. salmonis* are able to use semiochemicals excreted by Atlantic salmon to also make the best host selection, perhaps by choosing the least stressed host or a host secreting a compound indicating it is susceptible to infection.

### 2.6.2 *Lepeophtheirus salmonis* gene expression

No *L. salmonis* genes were found to be significantly differentially regulated during the 14 day cohabitation study between treatment groups. There was decreased variation in Trypsin-1, a digestive enzyme, in male lice that chose to move to a new host. This may be due to the increased energy required to seek and colonize a new host. Lice that remained on their initial host have had the opportunity to feed to satiation and may or may not need to be actively feeding which could lead to a large variation in trypsin secretion, while the lice on a new host may need to restore energy and expend energy reserves in order to suppress the activation of the immune system of the new host. Fast et al. (2002) have shown a decrease in macrophage function during infection of naive Atlantic salmon infected with pre-adult *L. salmonis* at 21 days post infection which they hypothesize may be caused by the feeding of the parasite. A number of low molecular weight proteases, thought to be trypsin-like, were also found in Atlantic salmon mucus. This finding corresponded to the decrease in macrophage function (Fast et al. 2002, 2003), which indicates that trypsin or other *L. salmonis* secretory proteins may play a role in regulating host immune responses.

### 2.6.3 Atlantic salmon gene expression

There was one instance of significant differential gene regulation in Atlantic salmon in this study. The expression of MMP9 was significantly higher in the spleen of the Initial Host at 2dpc as compared to the New Host at 2dpc. MMP9 is known to be involved in inflammation, having a role in regulating both the initiation and termination of this process (Chadzinska et al. 2008). It has been proposed to have a role in cell migration and tissue remodelling; Murakami and Mano (2006) have shown an increase in MMP9 expression after skin wounding in Japanese flounder. Using carp head kidney phagocytes Chadzinska et al. (2008) were also able to demonstrate a significant increase in MMP9 expression 4 to 24 hours after lipopolysaccharide (LPS) challenge. Skugor et al. (2008) previously demonstrated an increase in MMP9 expression in damaged skin tissue of salmon infected with *L. salmonis.* The low level of MMP9 expression observed in the spleen of New Hosts at 2dpc could indicate that while MMP9 is expressed to aid in tissue remodelling, it requires greater than 48 hours of stimulation by *L. salmonis* to be induced in the spleen. Although to mitigate damage caused by *L. salmonis* feeding MMP9 would need to be expressed in the skin, it is possible that it is only expressed very locally in skin tissues. *L. salmonis* adults move freely across the host as they feed, making it difficult to determine specific feeding site. As the fish in this study had not developed any lesions typical of heavy *L.*

*salmonis* infection, and a standardized section of skin was selected for gene expression analysis, changes in gene expression in the skin may have occurred and not been observed.

### 2.6.4 Salmon skin-spleen correlations

Due to the large number of correlations performed in this study, the opportunity for Type I errors to have occured was increased. To accomodate these potential errors, the authors have chosen to focus discussion on statistically significant correlations that have occured in multiple instances within a gene. In the New Host 2dpc treatment group both IL-1β and IL-8 expression in spleen tissue was positively correlated with IL-12 expression in the skin. This correlation is indicative of the progression of an initial inflammatory response, likely driven by IL-1β and IL-8 systemically, to a targeted $T_H1$ type response driven by IL-12, occuring in the skin or closer to the site of parasite attachment and feeding.

### 2.6.5 Correlations in host-parasite gene expression

The peroxinectin-like gene appears to have an important role in the relationship between *L. salmonis* and Atlantic salmon, being significantly correlated with nearly all of the markers of salmon immunity in the Initially Infected hosts. Peroxinectin is widely known to be an important mediator of the innate immune response of arthropods, being involved with cell adhesion, degranulation, promotion of encapsulation, opsonic, and peroxidase activities (Jiravanichpaisal et al. 2006). A review by Cerenius and Söderhäll (2004) suggests that peroxinectin may also have a role in inducing the production of microbicidal compounds. The putative immune response function of the peroxinectin-like gene may indicate an *L. salmonis* response to an Atlantic salmon defensive or immune response, additionally, it could be a result of exposure to microbes consumed during feeding. The peroxinectin-like gene from males and females was positively correlated with IgT in host skin and spleen. While the function for IgT is unknown in terms of ectoparasitic copepod infection, it has been observed previously to increase in Atlantic salmon skin following *L. salmonis* infection (Tadiso et al. 2011). Humoral responses and the lack of cell mediated responses in general have also been described in previous work on *L. salmonis* infections of Atlantic salmon (Tadiso et al. 2011). This could be partially driven by parasitic modulation towards a $T_H2$ immune response in the host rather than activation of macrophages and cell mediated immunity associated with a $T_H1$ host response.

A second consideration, when trying to understand the effect of the peroxinectin-like compound might have on salmon gene regulation, is that *L. salmonis* consume some level of blood meal and secrete serine proteases onto the skin of the fish, potentially to aid with feeding (Boxaspen 2006). Peroxinectin is not known to be secreted by *L. salmonis*, but Arcà et al. (2005) have shown that a salivary peroxidase is expressed in the salivary glands of the female mosquito, *Anopheles gambiae*, while it is minimally expressed in the carcass of the female and whole body analysis of the male. Only female mosquitos consume blood , indicating that peroxidase activity of the peroxinectin-like gene may have an essential role in blood feeding (Arcà et al. 2005). Correlations between the expression of the peroxinectin-like gene by male lice and the host grew stronger over the course of the experiment in male lice, suggesting that length of time on the host was a significant factor regulationg the interactions between the host and parasite gene regulation. This was supported by a greater frequency of significant correlations occuring between the female louse peroxinectin-like gene and host responses at 2 dpc. This relationship was not maintained at 14 dpc, but could suggest that a threshold of infection by the parasite is required to affect host responses, since the female lice number was approximately 50% at 14 dpc of what it had been at 2 dpc.

The positive correlations between louse expression of the peroxinectin-like, glycene receptor α-like, and Trypsin-1 genes and salmon skin expression of IL-12 in the Initial Host groups is likely due to IL-12's role in the innate immune sytem, and it's ability to initiate adaptive immune responses. Interleukin-12 is known to induce the production of the inflammatory cytokine Interferon λ, an inflammatory protein involved in innate immune responses. The ability of IL-12 to help induce the differentiation of T cells into $T_H1$ Cells can begin the initiation of an adaptive immune response, as $T_H1$ cells and IL-12 can induce proliferation of antibody producing B cells (Trinchieri 2003). The long duration of infection of the initially infected fish, 70+ days, would be a chronic stimulation of the salmon's inflammatory and wound healing responses, and the developement of an adaptive immune response may be occuring. Trypsin-1's role as a secretatory molecule and the peroxinectin-like gene's potential role as a secretatory protein may be continuously stimulating the host immune system resulting in triggering of B and T-cell signalling and adaptive responses associated with IgT and IL-12.

The gene correlations examining the effects of high vs. low infection level revealed that fish with low infection levels tended to have positive correlations with *L. salmonis* gene expression, where the higher infection levels had more variation between the numbers of positive and negative interactions. The constant positive correlation at low lice density suggest a uni-directional and potentially stimulatory effect of lice infection, with stronger impacts on the skin. However, the results of the high infection group are suggestive of a more dynamic regulation at both the site of the infection and spleen which may be the result of parasite influence with the host response

## 2.7 Conclusions

Interleukin-1, IL-8, IL-12, IgT and MMP9, expression in Atlantic salmon skin all appear to be important during lice infection, and re-infection (Purcell et al. 2013). Interleukin-1, IL-12, IgT and MMP9 were only significantly correlated with the peroxinectin-like gene in initially infected fish, suggesting Atlantic salmon may take some time to become responsive to *L. salmonis* infection. This would agree with previous research which demonstrated a significant increase in IL-12 in the skin of previously infected animals during CpG stimulation at 17 days post exposure and not at 7 days post exposure (Purcell et al. 2013). Interleukin-12's role as a $T_H1$ cytokine produced by antigen presenting cells and lymphocytes would suggest it may take longer to be stimulated and therefore measured. Furthermore, host response effects on louse gene expression may only become apparent after prolonged exposure to the louse and the 14 day exposure period to initially uninfected fish may not have been long enough to observe this effect. In most cases, *L. salmonis* gene expression had greater significant correlation with host expression over time, also suggesting that the host immune response itself took time to affect *L. salmonis* gene regulation.

This study highlights the importance of measuring both host and pathogen responses to each other at the individual level. In cases such as this, where out bred host and parasite populations are being used, individual variation may be quite high and make statistically significant observations across treatment groups difficult. By looking at host and parasite responses corresponding to each other we may be able to identify more subtle, yet still very important mechanisms at work in the host-parasite relationship.

## 2.8 Acknowledgements

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# CHAPTER 3

## USE OF AN *IN VITRO* BIOASSAY TO ASSESS CHANGES IN BEHAVIOUR AND GENE EXPRESSION OF *LEPEOPHTHEIRUS SALMONIS* DURING EXPOSURE TO BOTH HOST STIMULI AND AN ANTI-ATTACHMENT FACTOR

This chapter has been prepared as the following manuscript to be submitted for publication:

**Use of an *in vitro* bioassay to assess changes in behaviour and gene expression of *Lepeophtheirus salmonis* during exposure to both host stimuli and an anti-attachment factor**

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### 3.1 Abstract

*Lepeophtheirus salmonis,* the sea louse, is a crustacean parasite of salmonids, infection causes significant monetary damages and has negative impacts on fish health in the salmonid aquaculture industry. Due to the development of resistance to chemotherapeutants in the majority of *L. salmonis* populations, new methods for controlling infection levels need to be developed. The current study uses an *in vitro* exposure of copepodid and adult lice to both host cues and a purported anti-attachment factor, allyl isothiocyanate, to further investigate the effect of this compound on *L. salmonis.* Lice were exposed to a high, medium, and low dose of the anti-attachment factor for 6 and 12 hours, resulting in a trend of decreased observable fitness with increased dosage; lice in the high dose treatment rapidly became moribund. Adult lice were collected at both time points and underwent gene expression analysis of 7 genes associated with various facets of lice survival: CYP18 A1-like, cytochrome p450 isoform 1-like Protein, glycene receptor α-2-like, leukocyte receptor cluster member 9-like (LRCM9), nicotinic acetylcholine receptor subunit-like (nAChR-like subunit), peroxinectin-like, and Trypsin-1. It was noted that male lice had higher levels of expression in all genes compared to female lice, likely due to the dedication of a large portion of the female lice's body to reproduction. CYP18 A1-like gene and nAChR-like subunit gene demonstrated a dose dependent increase in gene expression, while Trypsin-1 and a LRCM9-like gene had a trend of increasing expression followed by a reduction in the higher doses. The trends in gene regulation indicated that exposures to the anti-attachment factor induced stress and possibly immunological pathways. Both behavioural observations and gene expression data suggests this compound would reduce survival and possibly settlement of the parasite.

### 3.2 Introduction

*Lepeophtheirus salmonis* is a copepod parasite found ubiquitously in northern marine waters (Verspoor, Stradmeyer, and Nielsen 2007). It parasitizes primarily salmonid fish, and has been noted to be particularly successful in infecting Atlantic salmon (Fast et al. 2002). As *L. salmonis* feed on salmon, large skin lesions can develop, resulting in susceptibility to secondary infections reduced feed conversion, and mortality. Damage caused by *L. salmonis* and other lice species costs the global aquaculture industry approximately $500M USD annually, attributed largely to reduced value of the final product and the cost of anti-sea lice chemotherapeutants (Costello 2009).

*L. salmonis* are ubiquitous in most salmon populations, they have been found on several potential reservoir hosts including wild salmonids, three spined stickleback (*Gasterosteus aculeatus*, Jones et al. 2006), sea bass (*Dicentrarchus labrax*, Pert, Urquhart and Bricknell 2006) saithe (*Pollachius virens*, Bruno and Stone 1990). It is likely that there will always be contact between *L. salmonis* and farmed Atlantic salmon. Current chemotherapeutants are used in response to increasing levels of lice infection and to mitigate the corresponding increase in damage to the fish. Dependence on chemotherapeutants has led to the development of drug resistance in lice populations around the world, which has necessitated the development of alternative control methods.

One tool that has been useful in the initial development of chemotherapeutants, monitoring of resistance, and testing to determine biological responses to stimuli is *in vitro* assays. This technique has been used to acquire information about many pest and parasite species. *In vitro* exposure of the cattle tick *Rhipicephalus microplus* to the pathogenic fungus *Metarhizium anisopliae* by Beys da Silva et al. (2010) was able to elucidate the role of extracellular lipase as a virulence factor. The secreted lipase is thought to play a role in adhesion of *M. anisopliae* to a host and in degrading of the top lipid layer of the tick cuticle to allow fungal propagules to infect the host. Completing the exposure in a controlled setting rather than analyzing samples of infected ticks from cattle allowed for the quantification of lipase activity over time and to confirm the role of lipase as an important virulence factor by use of a lipase-inhibited control group (Beys da Silva et al. 2010). Several *in vitro* assays have been developed to determine the effectiveness of compounds used to treat infection by the human scabies mite, *Sarcoptes scabiei* (Walton, Myerscough, and Currie 2000). Mites were exposed to the compound of interest by being placed in a Petri dish that had been coated on the top, bottom and sides with an emulsion of each treatment. These *in vitro* assays demonstrated the ability of benzyl benzoate, lindane, ivermectin and tea tree oils to kill mites within 3 hours, and showed increased permethrin lethality at 18 hours although it was not significantly different than the control samples (Walton, Myerscough, and Currie 2000). Bioassays have been an invaluable tool in monitoring the progression of resistance in sea lice as well. Following initial reports of treatment failures on salmon farms *in vitro* drug exposures were used to help determine if failure was caused by improper application or by drug resistance. Several studies have used short and long term exposures of sea lice to a chemotherapeutant to mimic either in feed or bath treatments (Saksida et al. 2013; Sevatdal and Horsberg 2003; Westcott et al. 2008). These assays have been useful in tracking development of resistance to a variety of drugs across different sea lice populations.

As many *L. salmonis* chemotherapeutants are derived from land-based agricultural applications, development of new therapeutants may also be derived from techniques adapted from land-based agriculture. An increasing amount of research has focused on the development of nature-derived treatments, adapting natural protective compounds from plant species. Allyl isothiocyanate is an organosulfur compound that can be extracted from mustard seed, horseradish, and wasabi (Wu et al. 2009). Used as a fumigant, allyl isothiocyanate causes mortality in the red flour beetle (*Tribolium castaneum*) and causes deformities during moulting, especially when exposed larvae and pupae moult to adult stages (Santos et al. 2011). Toxicity has also been observed in fumigation bioassays with *Sitophilus zeamais, Rhizoperta dominica, Tribolium ferrugineum,* and *Liposcelis entomophila,* with effective treatment doses ranging between 1.5-3µg/mL (Wu et al. 2009). Should *L. salmonis* respond to allyl isothiocyanate in a similar manner to other pest species, with interrupted moulting and mortality, allyl isothiocyanate may be a useful compound in management strategies.

Preliminary investigation of allyl isothiocyanate as a host masking or anti-attachment factor using Y-tube assays have been completed (EWOS Innovation, limited personal communication). The compound has been shown to suppress host seeking activity when *L. salmonis* is exposed to salmon conditioned water. The current study aims to elucidate some of the behavioural changes and differential gene expression that may occur during close contact with a salmonid host using an *in vitro* model designed to mimic salmon skin. This may provide evidence to support the effectiveness of this compound on host settling.

**3.3 Study Objectives**

Determine if exposure to an anti-attachment factor alters *L. salmonis* behaviour, survival, or gene expression in an *in vitro* model.

a. Monitor survival of copepodid and adult lice during exposure to an anti-attachment factor at increasing dosages.

    i. $H_i$: Allyl isothiocyanate will have a negative effect on lice survival

b. Use a plate bioassay to determine if differential gene expression occurs in copepodid or adult lice exposed to an anti-attachment factor.

    i. $H_i$: Allyl isothiocyanate will have a dose dependent effect on lice gene expression

**3.4 Materials and Methods**

**3.4.1 Lice collection and rearing**

Wild adult lice were collected from aquaculture farms in the Bay of Fundy region of NB, Canada, laboratory maintained lice populations were initially sourced from the same area. Laboratory maintained populations were raised from egg strings collected from wild gravid female lice. To ensure that the adult lice used in the study were healthy, weak lice were removed by mixing the water in the collection bucket and pouring off any lice not strong enough to remain attached to the sides of the bucket.

Copepodid lice were obtained by rearing egg strings collected from wild gravid female lice in hatch systems. An effort was made to exclude any abnormal egg strings from the rearing process. Hatch systems were filled with approximately 10 litres of sea water collected from the Bay of Fundy, NB, stored in a 13$^{o}$C incubator, and were constantly aerated using an aquarium pump. Two to three litres of the sea water in each system was replaced daily. Once 70-100% of nauplii had moulted to the copepodid stage they were collected for use in the *in vitro* assays.

**3.4.2 *In vitro* assays**

Three i*n vitro* assays were performed for both the adult and copepodid lice to determine if exposure to allyl isothiocyanate has an impact on lice survival and gene expression. An appropriate method of drug delivery needed to be developed. Previous research in our laboratory has shown that inclusion of salmon mucus in trypticase soy agar with salt (TSA*) media plates significantly increases association of copepodid and adult male lice by 5-10 fold, as compared to controls (Johnson, unpublished). This suggests that incorporation of mucus stimulates host finding or settlement behaviour. For this study we provided TSA* as an attachment substrate, and incorporated fish mucus as a host stimuli, and the anti-attachment factor, allyl isothiocyanate, into the substrate to attempt to elicit, then block host seeking activities in *L. salmonis.*

*In vitro* assays were performed in sterile disposable plastic Petri dishes. Attachment substrate in the Petri dishes was made by heating sterile TSA* in a microwave until melted. Once melted, the media was moved to a 50ºC water bath to keep warm. Working inside a biosafety cabinet, media was divided into four 90mL aliquots in sterile glass media bottles. Media bottles were labelled high (1ppt), medium (1ppm), low (1ppb) and 0ppb, and 100µL of anti-attachment factor was added to the high dose. The high dose was mixed by swirling the media around the bottle several times over the course of 5 minutes to allow the compound to thoroughly dissipate throughout the media. Following this, 100µL of the high dose media was transferred to the medium dose media. The medium dose was mixed in the same way as the high dose. Finally, 100µL of the medium dose media was transferred to the low dose, and mixed. Once all the drug doses had been made, 10mL of salmon mucus was added to each dose, quickly mixed by swirling the bottle, and poured onto Petri dishes. Salmon mucus used in the assays had been collected previously from Atlantic salmon uninfected with *L. salmonis* and stored at -80$^{O}$C. Approximately 16mL of each dose was poured onto triplicate plates for each time point. Plates were labelled with their dose, and allowed to set for a minimum of one hour at room temperature. *In vitro* assays were replicated three times for both copepodid and adult lice.

Bioassay set up differed between adult lice and copepodids. In adult lice assays, approximately 40mL of unfiltered natural sea water was added to each plate. One adult male and one adult female were added to each plate. In copepodid assays, approximately 50 copepodids were added to each plate. To accomplish this, excess sea water was filtered from the hatch systems using 100μm mesh, and the concentrated copepodids were counted to determine the number of copepodids per mL. Using this calculated concentration, enough volume was transferred to each Petri dish to ensure that 50 copepodids were added to each dish. Each dish was topped up to 40mL of liquid using unfiltered sea water collected from the Bay of Fundy area of NB, Canada. Samples of adult male, adult female and copepodids were collected and stored at -80$^{O}$C at this time in RNAlater® (Life Technologies, Carlsbad, California) as a time zero sample. Assay design is shown in Figure 3.1.

The Petri dishes of lice were incubated at 13$^{o}$C for six hours. At the six hour time point observations on lice health were made; observations of adult lice categorized each louse as being live, weak, moribund, or immobile (Table 3.1), and observations of copepodid lice aimed to be descriptive, commenting on movement and association with the substrate in the Petri dishes. After observation, all three lice from replicate Petri dishes of each treatment were collected and frozen at -80$^{O}$C in RNAlater. Adult lice were moved into collection tubes using forceps, while copepodids were filtered out of the incubation media using 100μm mesh and transferred into RNAlater. The remaining 3 replicates of each treatment were collected at the 12 hour time point. Once in RNAlater lice were stored at -80$^{o}$C for future use. Note that in the first copepodid *in vitro* assay a 24 hour time point was included, but removed from the study as the media had begun to dissociate.

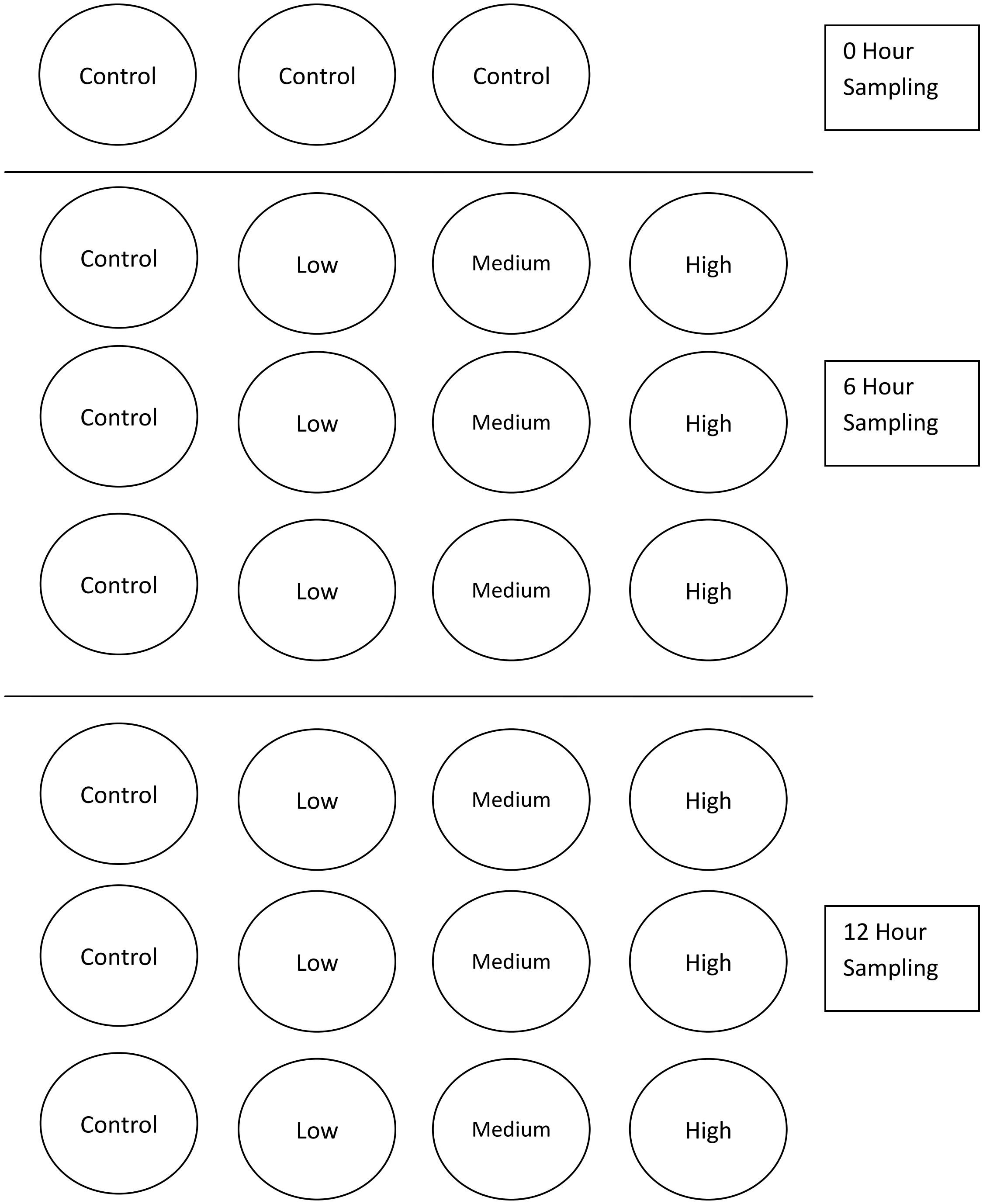



**Figure 3.1:** Schematic of *in vitro* assay design. Each circle represents a replicate Petri dish, dosage is noted within the circle. In adult assay each Petri dish contained one adult male and one adult female louse, while in copepodid assays each Petri dish contained ~50 copepodid lice.

**Table 3.1:** *Lepeophtheirus salmonis* health classifications (Adapted from Westcott et al., 2008)

| Response | Criteria |
|---|---|
| **Live** | (1) Normal swimming behaviour (ability to swim in a straight line)<br>(2) Securely adheres to Petri dish<br>(3) Normal movement of extremities |
| **Weak** | (1) Less vigorous swimming behaviour<br>(2) Adhere less strongly to Petri dish<br>(3) Normal movement of extremities |
| **Moribund** | (1) Disabled swimming, but capable of weak uncoordinated movement<br>(2) Inability to adhere firmly to Petri dish<br>(3) Minimal movement of extremities |
| **Immobile** | (1) Inability to swim<br>(2) Floating in Petri dish<br>(3) No movement of extremities |

Description of the observations used to describe the health status of adult male and female *L. salmonis.*

### 3.4.3 Gene expression

In an attempt to determine if exposure to allyl isothiocyanate was having a biological effect on *L. salmonis,* expression of 7 putative genes associated with different facets of louse survival were compared across treatment groups. In this series of experiments expression of CYP18 A1-like, Trypsin-1, Cytochrome p450 isoform-1 like protein, peroxinectin-like, leukocyte receptor cluster member 9-like (LRCM9), glycene receptor α-2 like (GRα-2), nicotinic acetylcholine receptor subunit-like (nAChS) were analysed. These genes have been previously studied with respect to host switching behaviour, as discussed in Chapter 2.

To obtain gene expression results, RNA was extracted from the collected lice. Briefly, whole adult lice and all collected copepodids underwent RNA extraction using a Tri-Reagent protocol. RNA purity and concentration was confirmed using a Nano Drop 2000 (Thermo Scientific, Wilmington, DE). Genomic DNA contamination was eliminated from samples using a DNase treatment (TURBO DNA-*free*™ kit, Ambion, Carlsbad, CA). Following DNase treatment, sample quality was assessed using a Bio-Rad Experion (Mississauga, ON); Copepodid RNA was degraded and copepodids were excluded from the remainder of the study. The RNA Quality Indicator values for adult lice were above 7 and considered to be of relatively high quality. cDNA was generated using a reverse transcription reaction (iScript™ Reverse Transcription Supermix for RT-qPCR, Bio-Rad, Hercules, CA); both DNase treatment and cDNA synthesis procedures followed manufacturer's instructions.

Quantitative PCR was performed using SsoAdvanced Sybr® Green Supermix (Bio-Rad, Hercules, CA). Reactions were performed using a three step protocol: One cycle of 95$^{o}$C for 10 minutes, forty cycles of 95$^{o}$C for 15 seconds, $T_{Annealing}$$^{o}$C for 15 seconds and 72$^{o}$C for 15 seconds, and one melt curve. $T_{Annealing}$ and curve efficiencies are described in Table 3.2. All samples were run for

**Table 3.2:** qPCR conditions for *Lepeophtheirus salmonis* genes

| Gene | Annealing Temperature (°C) | Standard Curve Efficiencies |
|---|---|---|
| **CYP18 A1-like** | 60 | 1.02-1.06 |
| **Trypsin-1** | 65 | 1.00-1.04 |
| **Cytochrome p450 Isoform-1 like Protein** | 65 | 1.05-1.08 |
| **Peroxinectin-like** | 60 | 0.98-1.03 |
| **Leukocyte receptor cluster member 9-like** | 65 | 0.98-1.04 |
| **Glycene receptor α-2 like** | 65 | 1.01 |
| **Nicotinic acetylcholine receptor subunit-like** | 65 | 0.98 |
| **Ribosomal Protein S20** | 61.0 | 0.99-1.01 |
| **Glyceraldehyde 3-phosphate dehydrogenase** | 65.1 | 0.99-1.04 |
| **Elongation Factor 1** | 65.1 | 0.96-1.01 |

qPCR conditions used to determine differential gene expression between treatment groups of *L. salmonis* exposed to varying concentrations of allyl isothiocyanate.

all 7 *L. salmonis* genes in duplicate, samples that could not be replicated to within 0.55 copy thresholds (cT) of each other were excluded from future analysis.  Three reference genes were included in the analysis; ribosomal protein S20, Glyceraldehyde 3-phosphate dehydrogenase and elongation factor 1.

Prior to statistical analysis, qPCR output must be normalized to account for variation that occurred while processing each sample.  The three reference genes were used to normalize the qPCR cTs of the genes of interest using qBase Plus software (Biogazelle, Zwiinaarde, Belgium). Within this software a geNorm analysis was included to ensure the stability of each of the reference genes.

### 3.4.4 Statistical analysis

Statistical analysis of MNRQ values were completed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA).  For each gene, within each treatment, samples with an MNRQ greater than two standard deviations from the mean were removed from their respective data set as an outlier.  The MNRQ values for each treatment group for each gene was checked to ensure the data set was normally distributed using either the D'Agostino & Pearson test or the Kolmogorov-Smirnov test for groups with small sample size.  If within a gene, more than one of the treatment groups was not normally distributed, the non-parametric Mann-Whitney test was used to determine if differential gene regulation was occurring between treatment groups.  The GraphPad Prism 6 software manual states that statistical tests are robust to slight deviations in normality, and for the purpose of this work 1/9, of the treatment groups being non-normally distributed is considered to be a slight deviation.  Normally distributed data was analysed using two-way ANOVA to determine if there were significant interactions between treatment dose and exposure duration; if there was no interaction between the two variables, the data from each time point (i.e. 6 hour control and 12 hour control) was combined and analysed using a one-way ANOVA with multiple comparisons using a Tukey's Test ($p<0.05$). Data sets where there was significant interaction between variable were analysed using a one-way ANOVA of all time point/treatment combinations.

## 3.5 Results

### 3.5.1 Behavioural observations

Observations of *L. salmonis* were made after 12 hours of incubation on the mucus-media plates (Table 3.3). Signs of normal activity in both copepodid lice and adult lice decreased as dosage of allyl isothiocyanate increased. It should be noted that immediately upon exposure to the high dose treatment the adult lice performed a vigorous spiral swimming pattern and appeared to immediately become immobile.

### 3.5.2 Gene expression

Gene expression analysis was completed for the adult lice only. During the execution of the copepodid allyl isothiocyanate bioassays the method of copepodid collection did not properly preserve the tissue. It is suspected that the amount of Tri-Reagent included in the RNA extraction protocol resulted in degraded RNA. For future studies of, it is recommended to store copepodid lice directly in Tri-Reagent.

Gene expression trends were observed between male and female adult lice, with male lice tending to have higher normalized expression for each gene of interest compared to female lice (Figure 3.2A-N).

**Table 3.3:** Behavioural observations of *L. salmonis* following a 12 hour incubation with an anti-attachment factor (allyl isothiocyanate)

| Treatment | Copepodids | Adults |
|---|---|---|
| **Control** | -Normal movement<br>-Associating with media | Live/Weak |
| **Low** | -Normal movement<br>-Associating with media | Live/Weak |
| **Medium** | -Less movement<br>-No association with media | Some morbidity |
| **High** | -No movement<br>-Pale colour | Immobile |

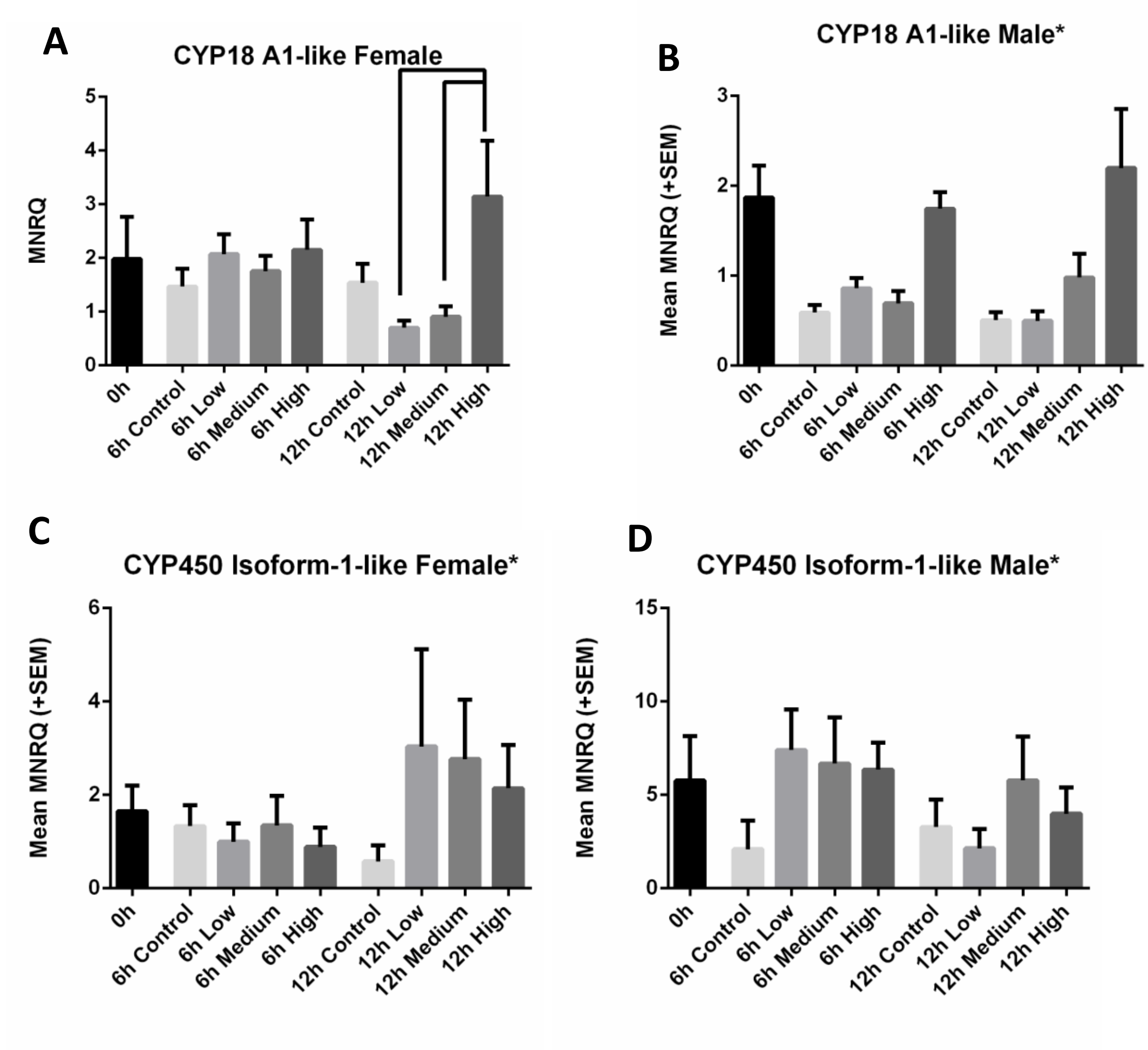
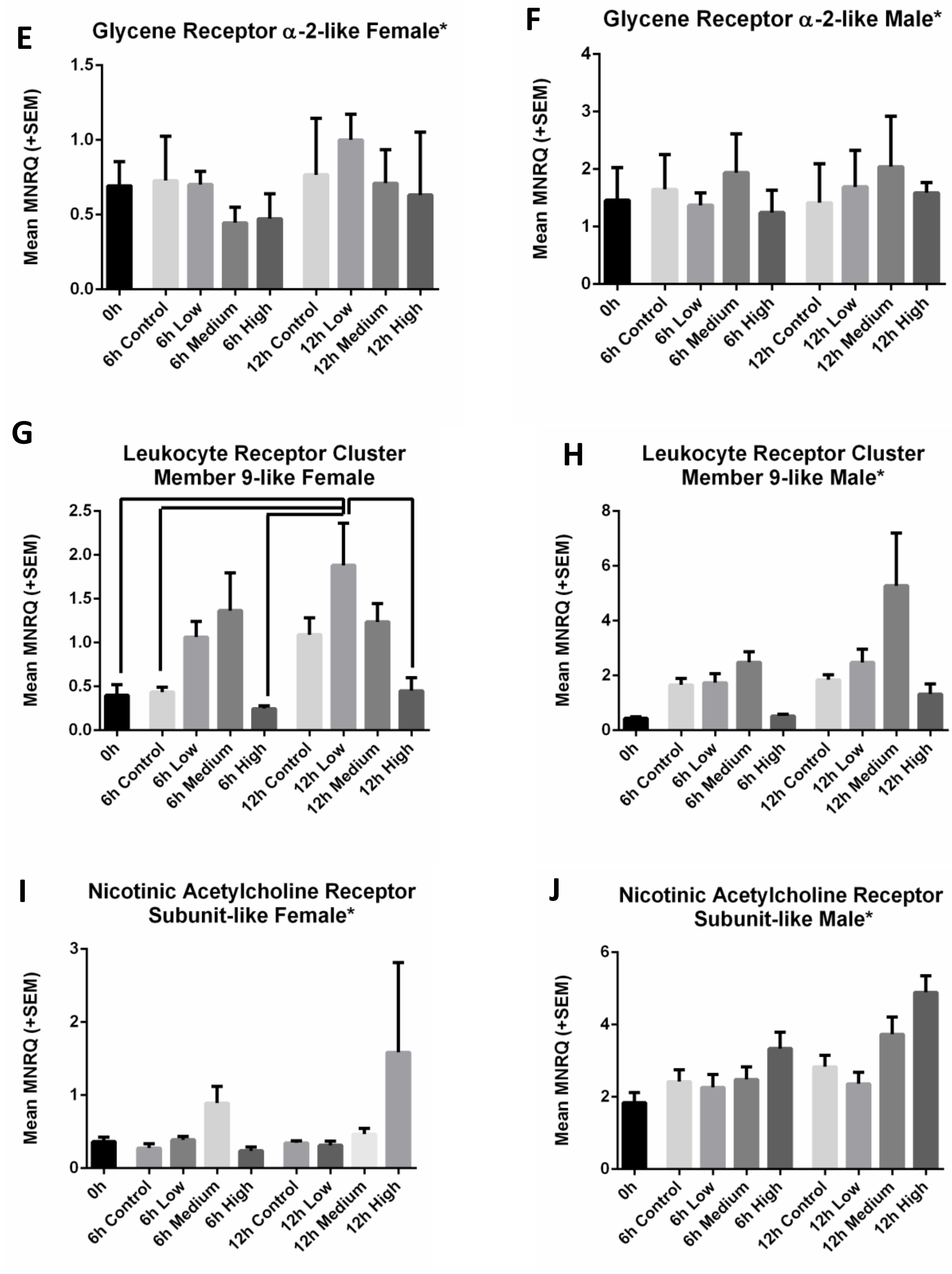
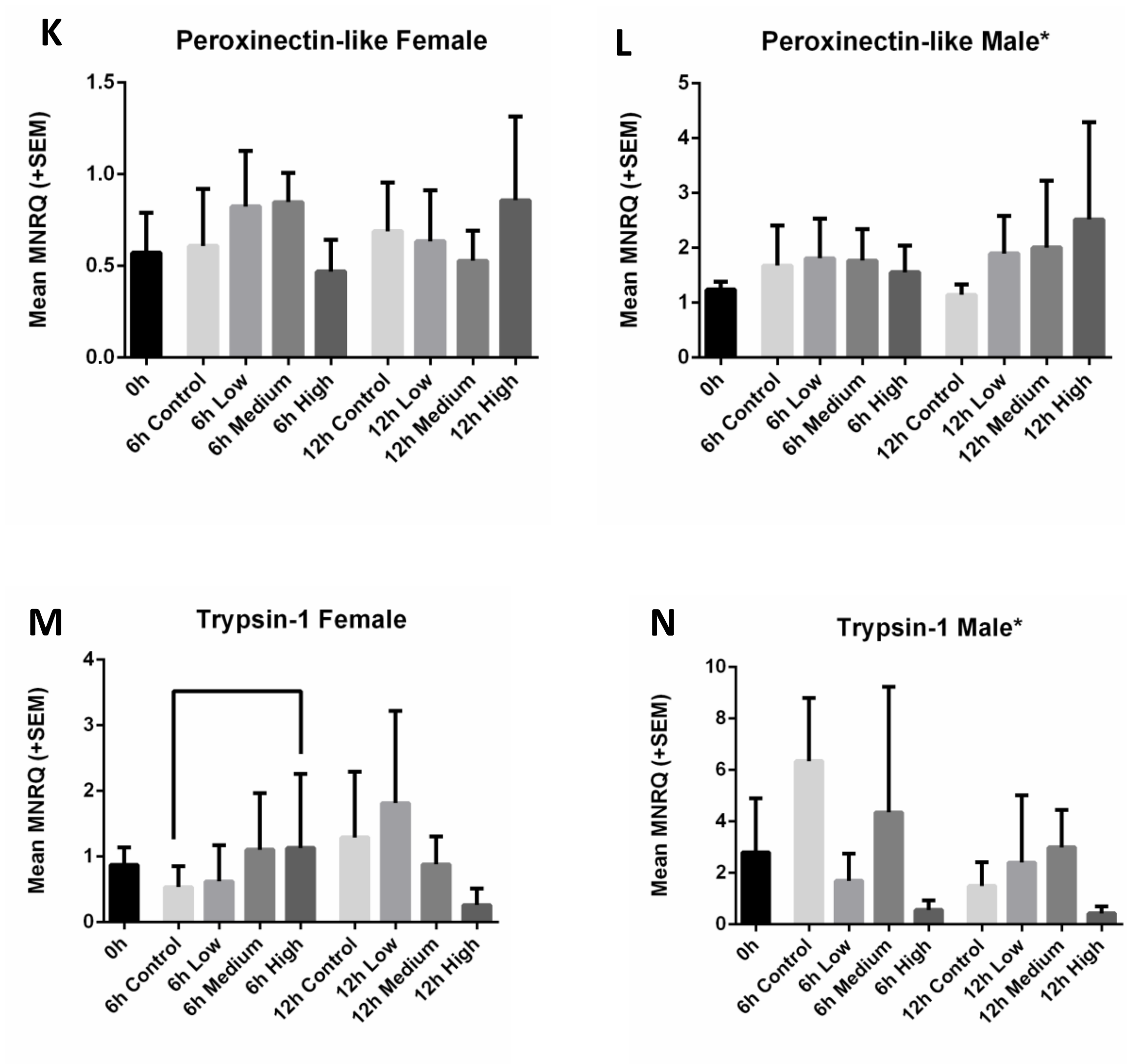



**Figure 3.2A-N:** Mean normalized relative quantity (MNRQ) (+SEM) of adult male and female *Lepeophtheirus salmonis* gene expression during exposure to a low (1ppb), medium (1ppm), high (1ppt) and control doses of allyl isothiocyanate over a 12 hour time period. *Figures marked by (*) were found to have no interaction between variable using a two-way ANOVA, and time points were combined for analysis with a one-way ANOVA, significant changes in expression between doses are described in the text.


E
Glycene Receptor α-2-like Female*
Mean MNRQ (+SEM)
1.5
1.0
0.5
0.0
0h
6h Control
6h Low
6h Medium
6h High
12h Control
12h Low
12h Medium
12h High
F
Glycene Receptor α-2-like Male*
Mean MNRQ (+SEM)
4
3
2
1
0
G
Leukocyte Receptor Cluster Member 9-like Female
Mean MNRQ (+SEM)
2.5
2.0
1.5
1.0
0.5
0.0
H
Leukocyte Receptor Cluster Member 9-like Male*
Mean MNRQ (+SEM)
8
6
4
2
0
I
Nicotinic Acetylcholine Receptor Subunit-like Female*
Mean MNRQ (+SEM)
3
2
1
0
J
Nicotinic Acetylcholine Receptor Subunit-like Male*
Mean MNRQ (+SEM)
6
4
2
0


K
Peroxinectin-like Female
Mean MNRQ (+SEM)
1.5
1.0
0.5
0.0
0h
6h Control
6h Low
6h Medium
6h High
12h Control
12h Low
12h Medium
12h High
L
Peroxinectin-like Male*
Mean MNRQ (+SEM)
5
4
3
2
1
0
0h
6h Control
6h Low
6h Medium
6h High
12h Control
12h Low
12h Medium
12h High
M
Trypsin-1 Female
Mean MNRQ (+SEM)
4
3
2
1
0
0h
6h Control
6h Low
6h Medium
6h High
12h Control
12h Low
12h Medium
12h High
N
Trypsin-1 Male*
Mean MNRQ (+SEM)
10
8
6
4
2
0
0h
6h Control
6h Low
6h Medium
6h High
12h Control
12h Low
12h Medium
12h High

Examining the gene expression of each gene in male and female lice resulted in one of 3 general responses: (1) No change in gene expression, (2) A dose dependent increase in gene expression, or (3) A dose dependent increase in gene expression followed by a reduction in expression in the higher doses. In female lice the CYP18 A1-like gene had the trend of a dose dependent increase in gene expression at the 12 hour time point, the 12 hour low and medium doses were statistically lower than the high dose (p=0.0205 and p=0.0375). CYP18 A1-like in male lice displayed a trend in dose dependent increase in expression at both 6 and 12 hours, the time 0 control was statistically higher than the control and low treatments (p=0.008 and p=0.0203). In female lice, the CYP450 isoform-1 like did not show any trends in expression at 6 hours and had a trend of increased expression followed by a reduction at 12 hours. In male lice the CYP450 isoform-1 like gene showed no consistent trends in expression. In female and male lice there were no consistent trends in expression of the GRα2-like gene. In female lice the LRCM9-like gene had a trend of dose dependent increase in gene expression followed by a reduction at the higher doses at both 6 and 12 hours, the time 0 sample, the 6 hour control and high doses and the 12 hour high dose were significantly lower than the 12 hour low dose (p=0.0192, p=0.0104, p=0.0036 and p=0.0274). In male lice the LRCM9-like gene had a trend of dose dependent increase in gene expression followed by a reduction at the higher doses at both 6 and 12 hours, the time 0 control was statistically lower than the medium dose (p=0.0093) and the control and high dose was statistically lower than the medium dose (p=0.0384 and p=0.0026). In the female lice there was a dose dependent trend in increased expression of the nAChR-like gene at 6 hours followed by a reduction in expression in the higher doses, at 12 hours there was a trend in dose dependent increase in expression. In male lice there were no consistent trends in expression of nAChR-like gene at 6 hours and there was dose dependent trend in increased gene expression at 12 hours, the time 0 control was statistically lower than the high dose (p=0.0011) and the control and low doses were statistically lower than the high dose(p= 0.0048 and p=0.0004). In female lice there was a trend of dose dependent increase in expression of the peroxinectin-like gene followed by reduction in expression at the higher doses at 6 hours and no consistent trends in expression at 12 hours. In male lice there were no consistent trends in the expression of the peroxinectin-like gene at 6 hours, and there was a trend of dose dependent increase in expression at 12 hours. Trypsin-1 in female lice had a trend of dose dependent increase at 6 hours, and a dose dependent increase followed by reduction at 12 hours, the 6 hour control was statistically lower than the high dose (p=0.0267). Trypsin-1 in male lice displayed no trends in gene expression at 6 hours, and had a trend of dose dependent increase followed by a reduction in gene expression at 12 hours.

Within the analysis of gene expression, several trends became clear; The CYP18-A1-like gene consistently had a trend of dose dependent increase in gene expression, at 12 hours both male and female lice exhibited the trend of dose dependent increase in Trypsin-1 gene expression followed by a reduction in the higher doses, the LRCM9-like gene consistently exhibited the trend of increased gene expression followed by a reduction in expression at the higher doses and finally, the nAChR-like exhibited a dose dependent increase in expression at 12 hours post exposure in both male and female lice.

**3.6 Discussion**

There are two possible causes for the higher levels of gene expression in male lice relative to female lice; (1) The male lice are actually expressing the gene at a higher level than females during the assay exposure, or (2) Expression of the measured genes is occurring in localized tissues and therefore, the relatively large genital segment and ovaries of the female lice is contributing to a dilution of these specific RNAs compared to the total RNA extracted from the sample. The genital segment of female lice has been shown to contain cement glands used to create the casing of the egg strings, maturing oocytes enclosed in egg strings, and spermatophore receptacles (Ritchie et al. 2012). The dedication of the female genital segment and ovary, which extends into the cephalothorax, to reproduction is likely to reduce the signal of the genes measured in this study, as the female genital segment can be nearly equal in size to the cephalothorax.

CYP18 A1 (Fig 3.2A) is thought to be an ecdysteroid agonist in the African cotton leafworm, *Spodoptera littoralis,* with a role in regulating the moulting process in insects (Davies et al. 2006). Expression of this gene increases most during the pharate pupal stage, when the adult structures of *S. littoralis* are formed (Davies et al. 2006). In the fruit fly, *Drosophila melanogaster,* knockdown of CYP18 A1 induces mortality during moulting, most prominently during the final moult from the pupal to adult stage. Appearance of melanisation is also observed in CYP18 A1 knockdown strains of *D. melanogaster* (Guittard et al. 2011). The observed upregulation of the CYP18 A1-like gene in adult female lice at 12 hours and the trend in upregulation in male lice at both time points suggest that the allyl isothiocyanate may be inducing expression of genes associated with moult and metamorphosis. Activation of these pathways in adult *L. salmonis* is considered unusual, as they have reached their final life stage and adult lice do not undergo further moults. In other crustaceans, several species of marine and land crab (*Gecarcinus lateralis*, *Callinectes sapidus*, *Carcinus maenas*, *Uca pugnax*, and *Uca pugilator*), precocious moulting can be induced through the removal of multiple limbs or eye stalks. Although the crab species studied continuously moult throughout their lives to accommodate growth, this provides evidence that the animals will moult earlier to recover from physical damage (Skinner et al. 1972).

A study on *L. salmonis* using a species specific 38K oligo microarray demonstrated that in a low salinity environment (10‰ salinity for a period of 24 hours) expression of several cuticle proteins in copepodid lice was also induced (Sutherland et al. 2012). Low salinities are lethal to *L. salmonis* and following exposure survivors were unable to colonize hosts at 4‰ salinity and 87.5% less capable of infecting a host at 12‰. This was thought to be largely caused by their inability to osmoregulate (Bricknell et al. 2006). Increased expression of moult associated proteins has thus been demonstrated as being a potential mechanism for responding to mechanical damage or adverse environmental conditions. Should the presence of allyl isothiocyante be an insult to *L. salmonis*, which was demonstrated by the observed morbidity and immobility that occurred during high dose exposure, increased expression of a moult-associated protein may have occurred in adult lice in an attempt to repair any damage that occurred.

Trypsin is known to be a secretory molecule associated with feeding and digestion in *L. salmonis* (Fast et al. 2003; Kvamme et al. 2004). *L. salmonis* have been shown to release trypsin onto their host and in media within 24 hours of stimulation with salmonid mucus (Fast et al. 2002; 2003). In this study the female lice tended to have an increase in the expression of Trypsin-1 compared to controls during the initial 6 hours of the bioassay exposure, and by 12 hours there was a trend in decreased expression in both the medium and high doses. The male lice showed a decrease in Trypsin-1 in the high dose at both 6 and 12 hour time points. The trend of increased expression may indicate an activation of feeding behaviour in response to the salmon mucus incorporated into the bioassay media, while the observed decrease in Trypsin-1 expression in the high dose treatment could be indicative of reduced palatability or feeding on the salmon mucus. Fast et al. (2003) showed a reduction in *L. salmonis* trypsin secretion in response to non-host mucus and in resistant salmonid species, which could indicate reduced feeding activity in the presence of unsuitable hosts. Trypsin-1 has been shown to be induced in mosquitoes after receiving either a sucrose or blood meal within 4 hours, peaking at 16-24 hours, and decreasing to basal expression by 40 hours (Dana et al. 2006). In the tick species *Ornithodoros moubata* an increase in the expression of lysozyme, an enzyme associated with digestion of food and microbes, is also induced beginning between 4 and 16 hours post feeding (Grunclova et al. 2003). The decreased expression of Trypsin-1 in the higher doses of allyl isothiocyanate indicates that the lice may be ceasing feeding due to the mucus now being unpalatable, stress, or incapacitation rendering them unable to feed.

A leukocyte receptor cluster member 9-like gene has been discovered in two species of sea lice, *L. salmonis* and *Caligus rogercresseyi*, both parasites of salmonid fish (Guo 2011, Koop, Pers. Com.). In this study the LRCM9-like gene exhibited a dose dependent increase in expression with increasing concentrations of allyl isothiocyanate followed by a reduction in expression in lice exposed to the higher doses. This gene is thought to be important during immune responses and is found to be highly expressed in macrophage enriched organs in mice (Guo et al. 2011). In mice, LRCM9 has a role in activating macrophage function, and the similarity of this molecule between mammals and the Chinese mitten crab, *Eriocheir sinensis*, suggests that the function of the gene in immune response activation may be conserved across mammals and arthropods. The precise role of LRCM9 in invertebrates, including *L. salmonis*, is unknown (Guo et al. 2011). The pattern of expression in this study may suggest that LRCM9-like is expressed as part of a general immune response, in this case initiated through the stress of exposure to the anti-attachment factor.

Finally, nicotinic acetylcholine receptor subunit (nAChR-like subunit)-like had a trend towards increased expression with exposure to increased concentration of the allyl isothiocyanate.

Nicotinic acetylcholine receptors are receptors of excitatory neurotransmitters, and have been used as a target for pesticide development in insects (Millar and Denholm 2007). Previous studies have shown upregulation of nAChR can be induced in cell lines using nicotine and neonicotinoid pesticides (Sallette et al. 2004; Tomizawa and Casida 2000). Nicotinic acetylcholine receptors are known to be important receptors in nociception, and allyl isothiocyanate has been shown to induce a pain response in mice (Jinks and Carstens 2013). Research in mice has shown that knockdown models of the β2 nAChR subunit lowers the pain threshold associated with both mechanical and thermal stimulation (Yalcin et al. 2011). Although pain perception in invertebrate species is debated, the glass prawn, *Palaemon elegans*, has been shown to exhibit heightened grooming response to noxious chemical stimuli. This heightened grooming is not a definitive proof of pain, but does demonstrate both the perception and reaction to adverse stimuli (Barr et al. 2008). The increase in the expression of a receptor important in pain perception in mammals coupled with the erratic swimming behaviour and morbidity observed in the high dose treatment with allyl isothiocyanate provides evidence to suggest that *L. salmonis* are responding to a noxious stimulus that may be causing the copepod to attempt to avoid the stimulus.

### 3.7 Conclusions

The combination of the observed behavioural and differential gene regulation that occurred during this study suggests allyl isothiocyanate causes a significant amount of stress to *L. salmonis*, possibly causing the initiation of an immune response (increased then decreased LRCM9-like gene), reduced feeding (decreased Trypsin-1), and a nociceptive response (increased nAChR-like). Morbidity was observed at the high dose treatment, which is not necessarily one of the intended endpoints and may not be an achievable concentration in any management strategy, but provides evidence to suggest that exposure to allyl isothiocyanate may induce avoidance behaviour, reduce potential settlement on the host, and ultimately have a negative impact on survival.  The changes in expression of the CYP18 A1-like gene could result in decreased survival of successive generations of lice feeding on salmon that had consumed allyl isothiocyanate by causing mortality during the moulting process.  Allyl isothiocyanate may be able to help reduce lice infection intensity in farmed Atlantic salmon by repelling adult lice through nociception and reducing fitness of successive generations.  In-feed trials are needed to confirm the effectiveness of allyl isothiocyanate as an anti-attachment factor, and inclusion of experiments to determine the survival rate of the early life stages of the parasite are needed to determine if survival during moulting is reduced.  Although several conclusions have been made in this study, it is important to note that this work is preliminary.  Lice were sourced from the same region, and relatively low numbers of lice were used in this study.

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# Chapter 4

## FINAL CONCLUSIONS AND FUTURE DIRECTIONS

## 4.1 Final Conclusions

*Lepeophtheirus salmonis* along with other species of sea lice, primarily *Caligus rogercresseyi* and *Caligus elongatus,* have been and continue to be a major pest in the salmonid aquaculture industry (Costello, 2009). Today, reliance on a small number of chemotherapeutants has driven the rapid development of resistance to these drugs (Atlantic Canada Fish Farmers Associations, 2012). Although there are several other drugs, such as cypermethrin, that have been shown to be capable of controlling sea lice infection, their use has been restricted due to high toxicity to both fish and crustaceans in the area surrounding aquaculture sites. The fluid nature of water moving through marine aquaculture sites makes it difficult to apply chemotherapeutants to cultured fish while limiting the spread and ensuing environmental impacts (Corner et al, 2008). Recently, an Atlantic Canadian company was ordered to pay a $500K penalty for releasing an illegal pesticide, Cypermethrin®, that resulted in mass lobster mortality, the largest fine ever issued in the region (Canadian Broadcasting Company, 2013). The damage caused to the lobster population, and the economic impact these mortalities can have on the lobster fishing industry, is motivation to develop sea lice treatments that more specifically target *L. salmonis.*

Understanding the transmission dynamics and molecular signals potentially associated with successful colonization of new hosts by *L. salmonis* could be an important tool in developing new treatment techniques that are specifically tailored to *L. salmonis.* Using a two week cohabitation of *L. salmonis* infected and uninfected salmon the present study (Chapter 2) demonstrated that adult male lice will rapidly reach an equal distribution across Atlantic salmon hosts. Female lice were unable, or chose not to move to a new host. This was thought to provide male lice with the highest likelihood of encountering a virgin female to mate with, and to minimize the risk the mature females would encounter trying to switch to a new host as 9 female lice were lost during the study. In Chapter 2, 8 *L. salmonis* genes, identified in a 38K oligonucleotide array as being differentially regulated during exposure to emamectin benzoate (Ben Koop, Pers. Com.), were selected to be analyzed based on their putative function. Five markers of Atlantic salmon immune status were also analyzed (Purcell et al. 2012). The study was unable to demonstrate differential gene regulation between the population of lice that moved to a new host and the population of lice that remained on their initial host. There was a significantly higher expression of MMP9 between the spleen of the initially infected host group at 2dpc compared to that of the initially uninfected group at 2dpc. This change in MMP9 expression is thought to be a result of the need for a period of time greater than 48 hours of infection being needed to stimulate production of enzymes in the spleen associated with wound healing in the spleen.

By examining the relationship between individual lice and their respective host, several instances of significant correlation were discovered, most interestingly between a peroxinectin-like gene in *L. salmonis* and IL-1β, IL-12 and MMP9 in the skin of Atlantic salmon. This correlation is suspected to be caused either due to peroxinectin secretions having a modulatory effect on the salmon immune system, or to the expression of salmon immune markers influencing the immune function of peroxinectin in the louse. There appears to be an increase in the incidence of correlation in fish with lower lice infection levels as compared to their more heavily inflected counterparts.

Use of an *in vitro* bioassay incorporating host stimuli and a purported anti-attachment factor, allyl isothiocyanate, was used to model the response of *L. salmonis* (Chapter 3). Adult male, adult female and copepodid lice were used in this assay, and subjected to 4 doses of the compound, 0ppt, 1ppt, 1ppm and 1ppb. Both copepodid and adult lice experienced increased morbidity in the higher treatment doses. Gene expression between treatment groups was examined in adult male and adult female lice. It was found that lice exhibited one of 3 trends in gene expression: 1) No change between groups, 2) A dose dependent increase in gene expression, and 3) An increase in gene expression in low doses followed by a reduction in gene expression in the higher doses. CYP18 A1-like, Trypsin-1, leukocyte receptor cluster member 9-like and nicotinic acetylcholine receptor subunit-like showed dose dependent changes in expression in both male and female lice. It is proposed in this work that the combination of genes being differentially regulated indicates the initiation of a general immune or stress response in *L. salmonis* when exposed to allyl isothiocyanate. The increase in CYP18 A1-like gene expression possibly indicates damage to the cuticle of the lice, the decrease in Trypsin-1 expression likely indicates a reduction or cessation of feeding during exposure, the decreased expression of the LRCM9-like gene at high doses could indicate an immune response and the increase in nAChR-like gene expression could indicate a nociceptive response.

### 4.2 Future Directions

There are several recommendations on modifications that may reveal additional information using or expanding the protocols that were used in Chapter 2 and 3.

#### 4.2.1 Cohabitation study

In the cohabitation study there is a question as to whether lice that switched hosts did so frequently, or switched hosts only once. This study made the assumption that male lice did not switch hosts more than once, or at least did not return to a previously infected fish, due to the high retention of lice in the study. It would be beneficial to confirm this assumption by individually labelling lice, however at this time no technique has been developed to allow lice to be labelled.

The initially infected salmon used in this cohabitation study did not have a high infection load, with only 4 lice/fish. In Atlantic Canada, aquaculture salmon often have much higher rates of infection (Atlantic Canadian Fish Farmers Association, 2012). With a final lice ratio of 2 lice/fish this study may be unable to observe changes in gene expression that occur in fish and lice when industry levels (Atlantic Canada) of infection are achieved. Additionally, fish in this study did not develop the lesions characteristic of high levels of *L. salmonis.* Repeating this study with a higher number of initial lice may amplify changes that occur between the different treatment groups, and allow observations to be made on changes that occur during lesion formation.

**4.2.2 Allyl isothiocyanate bioassays**

For future studies of, it is recommended to store copepodid lice directly in Tri-Reagent.

The bioassay experiments provided evidence to support allyl isothiocyanate as having both behavioural and gene regulation impacts on *L*. salmonis. Additional bioassays including lice sourced from a variety of regions and higher numbers would strengthen these findings. While this is a promising first piece of evidence, *in vivo* experiments need to be completed to determine if biologically relevant amounts of this compound will reach the skin of Atlantic salmon fed an allyl isothiocyanate supplemented feed. Work is being done to initiate an *in vivo* study of this nature in the Hoplite research group.

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## Appendix A – Pearson correlation data examining Atlantic salmon skin and spleen tissues

**Table A-1** Pearson correlations between expression of salmon immune markers in salmon skin and spleen tissues across all time points in a 14 day cohabitation study.

Skin vs. Spleen: All Time Points

| | IL-1β Skin | | IL-8 Skin | | IL-12 Skin | | IgT Skin | | MMP9 Skin | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value |
| IL-1β Spleen | 0.346 | 0.029 | -0.181 | 0.276 | 0.407 | 0.012 | -0.043 | 0.796 | 0.058 | 0.724 |
| IL-8 Spleen | -0.261 | 0.113 | -0.039 | 0.820 | 0.156 | 0.372 | -0.071 | 0.680 | 0.049 | 0.768 |
| IL-12 Spleen | -0.153 | 0.344 | -0.115 | 0.493 | 0.057 | 0.739 | 0.110 | 0.512 | 0.110 | 0.501 |
| IgT Spleen | -0.320 | 0.044 | -0.225 | 0.175 | -0.126 | 0.458 | 0.228 | 0.169 | -0.133 | 0.413 |
| MMP9 Spleen | 0.137 | 0.401 | 0.019 | 0.911 | 0.042 | 0.805 | 0.051 | 0.761 | -0.259 | 0.107 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of five markers of salmon immune response in *Lepeophtheirus salmonis* infected Atlantic salmon skin and spleen tissues across all time points and infection status during a 14 day cohabitation study of salmon initially infected and initially uninfected with *L. salmonis*. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table A-2** Pearson correlations between expression of salmon immune markers in salmon skin and spleen tissues in initially infected salmon 2 days post cohabitation.

Skin vs. Spleen: Initial Host 2dpc

| | IL-1β Skin | | IL-8 Skin | | IL-12 Skin | | IgT Skin | | MMP9 Skin | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value |
| IL-1β Spleen | 0.162 | 0.728 | 0.593 | 0.215 | 0.098 | 0.835 | -0.666 | 0.103 | 0.111 | 0.813 |
| IL-8 Spleen | -0.178 | 0.673 | 0.208 | 0.655 | 0.395 | 0.333 | -0.636 | 0.090 | -0.174 | 0.680 |
| IL-12 Spleen | -0.776 | 0.024 | 0.081 | 0.863 | -0.033 | 0.937 | -0.033 | 0.939 | -0.654 | 0.079 |
| IgT Spleen | -0.379 | 0.355 | 0.584 | 0.169 | -0.314 | 0.448 | -0.415 | 0.306 | -0.187 | 0.658 |
| MMP9 Spleen | -0.018 | 0.966 | -0.476 | 0.280 | 0.339 | 0.411 | 0.443 | 0.272 | -0.514 | 0.192 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of five markers of salmon immune response in *Lepeophtheirus salmonis* infected Atlantic salmon skin and spleen tissues in initially infected salmon on day 2 of a 14 day cohabitation study of salmon initially infected and initially uninfected with *L. salmonis*. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table A-3** Pearson correlations between expression of salmon immune markers in salmon skin and spleen tissues in initially infected salmon 14 days post cohabitation.

Skin vs. Spleen: Initial Host 14dpc

| | IL-1β Skin | | IL-8 Skin | | IL-12 Skin | | IgT Skin | | MMP9 Skin | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value |
| IL-1β Spleen | 0.424 | 0.296 | -0.220 | 0.601 | -0.027 | 0.954 | 0.034 | 0.942 | 0.120 | 0.778 |
| IL-8 Spleen | 0.582 | 0.130 | 0.298 | 0.473 | 0.603 | 0.152 | -0.050 | 0.915 | 0.721 | 0.043 |
| IL-12 Spleen | -0.170 | 0.662 | -0.043 | 0.913 | -0.055 | 0.898 | -0.240 | 0.567 | 0.276 | 0.472 |
| IgT Spleen | -0.655 | 0.055 | -0.227 | 0.558 | -0.327 | 0.429 | 0.268 | 0.522 | -0.385 | 0.307 |
| MMP9 Spleen | -0.636 | 0.090 | -0.072 | 0.865 | -0.248 | 0.592 | -0.013 | 0.976 | 0.008 | 0.984 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of five markers of salmon immune response in *Lepeophtheirus salmonis* infected Atlantic salmon skin and spleen tissues in initially infected salmon on day 14 of a 14 day cohabitation study of salmon initially infected and initially uninfected with *L. salmonis*. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table A-4** Pearson correlations between expression of salmon immune markers in salmon skin and spleen tissues in initially uninfected salmon 2 days post cohabitation.

Skin vs. Spleen: New Host 2dpc

| | IL-1β Skin | | IL-8 Skin | | IL-12 Skin | | IgT Skin | | MMP9 Skin | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value |
| IL-1β Spleen | 0.359 | 0.309 | 0.455 | 0.219 | 0.760 | 0.029 | -0.148 | 0.704 | -0.526 | 0.118 |
| IL-8 Spleen | 0.299 | 0.472 | 0.019 | 0.967 | 0.865 | 0.026 | -0.726 | 0.065 | -0.290 | 0.485 |
| IL-12 Spleen | 0.289 | 0.418 | 0.348 | 0.359 | 0.188 | 0.655 | 0.241 | 0.532 | -0.630 | 0.051 |
| IgT Spleen | -0.048 | 0.896 | -0.276 | 0.472 | -0.178 | 0.673 | 0.145 | 0.710 | -0.340 | 0.336 |
| MMP9 Spleen | -0.576 | 0.104 | -0.680 | 0.064 | -0.461 | 0.298 | -0.916 | 0.001 | 0.408 | 0.275 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of five markers of salmon immune response in *Lepeophtheirus salmonis* infected Atlantic salmon skin and spleen tissues in initially uninfected salmon on day 2 of a 14 day cohabitation study of salmon initially infected and initially uninfected with *L. salmonis*. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table A-5** Pearson correlations between expression of salmon immune markers in salmon skin and spleen tissues in initially uninfected salmon 14 days post cohabitation.

Skin vs. Spleen: New Host 14dpc

| | IL-1β Skin | | IL-8 Skin | | IL-12 Skin | | IgT Skin | | MMP9 Skin | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value |
| IL-1β Spleen | 0.781 | 0.008 | -0.140 | 0.720 | -0.194 | 0.567 | -0.140 | 0.700 | -0.022 | 0.949 |
| IL-8 Spleen | -0.511 | 0.160 | 0.274 | 0.511 | -0.257 | 0.473 | -0.009 | 0.982 | 0.207 | 0.566 |
| IL-12 Spleen | -0.204 | 0.571 | -0.397 | 0.290 | -0.073 | 0.832 | 0.428 | 0.217 | 0.269 | 0.423 |
| IgT Spleen | -0.172 | 0.634 | 0.062 | 0.875 | 0.187 | 0.582 | -0.002 | 0.996 | -0.005 | 0.988 |
| MMP9 Spleen | -0.395 | 0.258 | 0.408 | 0.275 | 0.455 | 0.187 | 0.013 | 0.974 | 0.089 | 0.807 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of five markers of salmon immune response in *Lepeophtheirus salmonis* infected Atlantic salmon skin and spleen tissues in initially uninfected salmon on day 14 of a 14 day cohabitation study of salmon initially infected and initially uninfected with *L. salmonis*. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Appendix B – Pearson correlations of *Lepeophtheirus salmonis* gene expression compared to gene expression of Atlantic salmon immunological markers in both skin and spleen tissue**

**Table B-1** Pearson correlations between the expression of salmon immune markers in salmon skin and expression of 8 genes in male *Lepeophtheirus salmonis* across all time points of a 14 day cohabitation study.

Male Lice vs. Skin: All Time Points

| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value |
| CYP18 A1-like | 0.060 | 0.719 | -0.153 | 0.381 | 0.048 | 0.780 | -0.108 | 0.530 | -0.129 | 0.433 |
| **CYP450** I-1 like | -0.202 | 0.321 | -0.207 | 0.344 | 0.209 | 0.338 | -0.285 | 0.167 | -0.010 | 0.961 |
| GRα-2-like | -0.173 | 0.299 | -0.324 | 0.058 | -0.137 | 0.425 | -0.136 | 0.430 | -0.192 | 0.243 |
| LRCM9 | 0.034 | 0.843 | -0.131 | 0.461 | -0.245 | 0.155 | 0.310 | 0.070 | 0.039 | 0.815 |
| nAChR subunit | 0.153 | 0.360 | -0.083 | 0.636 | -0.133 | 0.441 | 0.058 | 0.738 | -0.025 | 0.881 |
| Peroxinectin-like | -0.175 | 0.292 | -0.357 | 0.036 | -0.280 | 0.094 | 0.041 | 0.810 | -0.208 | 0.203 |
| TPAP | -0.107 | 0.565 | 0.149 | 0.449 | 0.141 | 0.458 | 0.297 | 0.111 | -0.167 | 0.361 |
| Trypsin 1 | 0.031 | 0.859 | -0.142 | 0.430 | 0.017 | 0.923 | 0.028 | 0.875 | -0.039 | 0.820 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult male *Lepeophtheirus salmonis* genes and the MNRQ of five markers of Atlantic salmon immune response in skin tissue across all time points of a 14 day cohabitation of Atlantic salmon initially infected and uninfected with *L. salmonis*. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with $p>0.05$ are significantly different than 0.

**Table B-2** Pearson correlations between the expression of salmon immune markers in salmon skin in initially infected fish and expression of 8 genes in male *Lepeophtheirus salmonis* on day 2 of a 14 day cohabitation study.

Male Lice vs. Skin: Initial Host 2dpc

| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value |
| CYP18 A1-like | -0.141 | 0.697 | -0.153 | 0.694 | -0.100 | 0.783 | -0.551 | 0.099 | -0.117 | 0.747 |
| CYP450 I-1 like | -0.389 | 0.340 | -0.844 | 0.017 | -0.319 | 0.441 | -0.046 | 0.914 | -0.465 | 0.245 |
| GRα-2-like | -0.025 | 0.946 | 0.480 | 0.191 | 0.020 | 0.955 | -0.440 | 0.203 | 0.197 | 0.586 |
| LRCM9 | -0.109 | 0.765 | 0.120 | 0.758 | -0.447 | 0.195 | 0.392 | 0.262 | -0.122 | 0.738 |
| nAChR subunit | 0.190 | 0.624 | 0.717 | 0.045 | 0.486 | 0.185 | 0.168 | 0.665 | 0.423 | 0.257 |
| Peroxinectin-like | -0.025 | 0.944 | -0.498 | 0.173 | 0.049 | 0.894 | 0.641 | 0.046 | -0.399 | 0.254 |
| TPAP-like | -0.376 | 0.319 | 0.018 | 0.965 | 0.501 | 0.170 | 0.626 | 0.071 | -0.495 | 0.176 |
| Trypsin 1 | -0.052 | 0.894 | 0.075 | 0.860 | -0.420 | 0.260 | -0.145 | 0.710 | -0.224 | 0.563 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult male *Lepeophtheirus salmonis* genes and the MNRQ of five markers of Atlantic salmon immune response in skin tissue on day 2 of a 14 day cohabitation of Atlantic salmon initially infected with *L. salmonis*. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table B-3** Pearson correlations between the expression of salmon immune markers in salmon skin in initially infected fish and expression of 8 genes in male *Lepeophtheirus salmonis* on day 14 of a 14 day cohabitation study.

| Male Lice vs. Skin: Initial Host 14dpc | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
| | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value |
| CYP18 A1-like | 0.587 | 0.097 | 0.171 | 0.685 | 0.200 | 0.605 | -0.558 | 0.193 | 0.701 | 0.035 |
| CYP450 I-1 like | 0.073 | 0.891 | 0.539 | 0.349 | 0.718 | 0.172 | -0.717 | 0.173 | 0.303 | 0.560 |
| GRα-2-like | -0.150 | 0.678 | -0.250 | 0.517 | 0.066 | 0.867 | 0.208 | 0.621 | -0.436 | 0.207 |
| LRCM9-like | 0.048 | 0.902 | -0.560 | 0.149 | -0.119 | 0.779 | 0.674 | 0.097 | -0.196 | 0.613 |
| nAChR-like subunit | 0.137 | 0.705 | 0.149 | 0.702 | 0.298 | 0.436 | -0.532 | 0.174 | 0.379 | 0.280 |
| Peroxinectin-like | -0.759 | 0.011 | -0.657 | 0.054 | -0.533 | 0.140 | 0.726 | 0.042 | -0.436 | 0.208 |
| TPAP | 0.133 | 0.777 | 0.007 | 0.989 | -0.572 | 0.235 | -0.523 | 0.366 | 0.313 | 0.494 |
| Trypsin 1 | 0.328 | 0.390 | -0.288 | 0.490 | 0.033 | 0.938 | 0.116 | 0.805 | -0.076 | 0.845 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult male *Lepeophtheirus salmonis* genes and the MNRQ of five markers of Atlantic salmon immune response in skin tissue on day 14 of a 14 day cohabitation of Atlantic salmon initially infected with *L. salmonis*. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed in *L. salmonis*. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table B-4** Pearson correlations between the expression of salmon immune markers in salmon skin in initially uninfected fish and expression of 8 genes in male *Lepeophtheirus salmonis* on day 2 of a 14 day cohabitation study.

| Male Lice vs. Skin: New Host 2dpc | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
| | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value |
| CYP18 A1-like | -0.190 | 0.600 | -0.195 | 0.590 | -0.082 | 0.846 | 0.245 | 0.496 | -0.417 | 0.231 |
| CYP450 I-1 like | -0.066 | 0.901 | -0.170 | 0.747 | 0.069 | 0.931 | -0.326 | 0.528 | 0.410 | 0.419 |
| GRα-2-like | -0.388 | 0.268 | -0.530 | 0.115 | -0.350 | 0.395 | -0.331 | 0.350 | 0.499 | 0.142 |
| LRCM9-like | 0.083 | 0.820 | 0.223 | 0.536 | -0.299 | 0.471 | 0.361 | 0.306 | 0.592 | 0.072 |
| nAChR-like subunit | 0.130 | 0.721 | 0.287 | 0.422 | -0.310 | 0.454 | 0.285 | 0.425 | 0.028 | 0.938 |
| Peroxinectin-like | -0.358 | 0.344 | -0.321 | 0.400 | -0.084 | 0.844 | -0.453 | 0.221 | -0.219 | 0.572 |
| TPAP | 0.065 | 0.867 | 0.045 | 0.909 | 0.359 | 0.382 | 0.126 | 0.748 | -0.396 | 0.291 |
| Trypsin 1 | -0.013 | 0.973 | 0.071 | 0.856 | 0.088 | 0.851 | 0.371 | 0.325 | 0.415 | 0.266 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult male *Lepeophtheirus salmonis* genes and the MNRQ of five markers of Atlantic salmon immune response in skin tissue on day 2 of a 14 day cohabitation of Atlantic salmon initially uninfected with *L. salmonis*. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed in *L. salmonis*. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table B-5** Pearson correlations between the expression of salmon immune markers in salmon skin in initially uninfected fish and expression of 8 genes in male *Lepeophtheirus salmonis* on day 14 of a 14 day cohabitation study.

Male Lice vs. Skin: New Host 14dpc

| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value |
| CYP18 A1-like | 0.222 | 0.565 | -0.213 | 0.613 | 0.364 | 0.301 | -0.618 | 0.076 | -0.639 | 0.047 |
| CYP450 I-1 like | 0.007 | 0.989 | -0.086 | 0.891 | 0.283 | 0.586 | -0.762 | 0.078 | -0.564 | 0.243 |
| GRα-2-like | -0.199 | 0.636 | 0.188 | 0.686 | 0.031 | 0.937 | -0.118 | 0.781 | -0.507 | 0.164 |
| LRCM9-like | 0.151 | 0.721 | -0.573 | 0.179 | 0.036 | 0.926 | -0.655 | 0.078 | -0.355 | 0.348 |
| nAChR-like subunit | 0.383 | 0.309 | -0.589 | 0.125 | -0.511 | 0.132 | -0.461 | 0.211 | -0.527 | 0.117 |
| Peroxinectin-like | 0.603 | 0.086 | -0.097 | 0.820 | -0.582 | 0.078 | -0.349 | 0.357 | -0.284 | 0.427 |
| TPAP | -0.719 | 0.108 | 0.671 | 0.215 | -0.741 | 0.057 | 0.348 | 0.444 | 0.090 | 0.847 |
| Trypsin 1 | -0.499 | 0.171 | -0.101 | 0.811 | 0.641 | 0.046 | 0.196 | 0.614 | -0.138 | 0.704 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult male *Lepeophtheirus salmonis* genes and the MNRQ of five markers of Atlantic salmon immune response in skin tissue on day 14 of a 14 day cohabitation of Atlantic salmon initially uninfected with *L. salmonis*. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed in *L. salmonis*. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table B-6** Pearson correlations between the expression of salmon immune markers in salmon spleen and expression of 8 genes in male *Lepeophtheirus salmonis* across all time points of a 14 day cohabitation study.

Male Lice vs. Spleen: All Time Points

| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value |
| CYP18 A1-like | -0.088 | 0.610 | 0.028 | 0.872 | 0.136 | 0.409 | 0.054 | 0.744 | -0.094 | 0.584 |
| CYP450 I-1 like | -0.260 | 0.220 | 0.070 | 0.740 | 0.098 | 0.634 | 0.142 | 0.489 | -0.108 | 0.609 |
| GRα-2-like | 0.058 | 0.738 | 0.135 | 0.440 | -0.092 | 0.579 | 0.137 | 0.405 | -0.009 | 0.958 |
| LRCM9-like | 0.175 | 0.315 | -0.228 | 0.194 | 0.138 | 0.409 | 0.252 | 0.126 | -0.189 | 0.276 |
| nAChR-like subunit | 0.193 | 0.258 | -0.259 | 0.132 | 0.117 | 0.479 | 0.105 | 0.524 | -0.383 | 0.021 |
| Peroxinectin-like | 0.169 | 0.325 | -0.160 | 0.358 | -0.094 | 0.570 | -0.106 | 0.520 | 0.016 | 0.925 |
| TPAP | -0.086 | 0.652 | 0.108 | 0.579 | -0.048 | 0.796 | -0.195 | 0.285 | 0.347 | 0.066 |
| Trypsin 1 | 0.028 | 0.874 | 0.291 | 0.095 | 0.260 | 0.120 | 0.129 | 0.445 | 0.270 | 0.122 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult male *Lepeophtheirus salmonis* genes and the MNRQ of five markers of Atlantic salmon immune response in spleen tissue across all time points of a 14 day cohabitation of Atlantic salmon initially infected and uninfected with *L. salmonis*. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table B-7** Pearson correlations between the expression of salmon immune markers in salmon spleen in initially infected fish and expression of 8 genes in male *Lepeophtheirus salmonis* on day 2 of a 14 day cohabitation study.

Male Lice vs. Spleen: Initial Host 2dpc

| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value |
| CYP18 A1-like | 0.252 | 0.547 | 0.579 | 0.079 | 0.154 | 0.671 | 0.274 | 0.444 | -0.072 | 0.844 |
| CYP450 I-1 like | -0.492 | 0.322 | -0.108 | 0.798 | 0.138 | 0.744 | -0.023 | 0.956 | -0.308 | 0.458 |
| GRα-2-like | 0.434 | 0.283 | 0.371 | 0.292 | 0.197 | 0.585 | 0.407 | 0.243 | -0.235 | 0.513 |
| LRCM9-like | 0.167 | 0.693 | -0.398 | 0.255 | 0.414 | 0.234 | 0.482 | 0.158 | -0.012 | 0.973 |
| nAChR-like subunit | -0.210 | 0.651 | -0.328 | 0.390 | -0.354 | 0.350 | -0.421 | 0.259 | -0.176 | 0.651 |
| Peroxinectin-like | -0.125 | 0.769 | -0.245 | 0.495 | 0.012 | 0.973 | -0.218 | 0.546 | 0.607 | 0.063 |
| TPAP | 0.143 | 0.760 | 0.134 | 0.730 | 0.281 | 0.464 | -0.025 | 0.949 | 0.679 | 0.044 |
| Trypsin 1 | 0.653 | 0.112 | 0.340 | 0.371 | 0.566 | 0.113 | 0.736 | 0.024 | 0.337 | 0.375 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult male *Lepeophtheirus salmonis* genes and the MNRQ of five markers of Atlantic salmon immune response in spleen tissue on day 2 of a 14 day cohabitation of Atlantic salmon initially infected with *L. salmonis*. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table B-8** Pearson correlations between the expression of salmon immune markers in salmon spleen in initially infected fish and expression of 8 genes in male *Lepeophtheirus salmonis* on day 14 of a 14 day cohabitation study.

Male Lice vs. Spleen: Initial Host 14dpc

| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value |
| CYP18 A1-like | -0.469 | 0.242 | -0.021 | 0.961 | 0.042 | 0.914 | -0.675 | 0.046 | -0.422 | 0.297 |
| CYP450 I-1 like | -0.162 | 0.758 | 0.271 | 0.603 | 0.116 | 0.826 | 0.244 | 0.641 | 0.649 | 0.163 |
| GRα-2-like | 0.250 | 0.516 | 0.389 | 0.300 | 0.311 | 0.381 | 0.298 | 0.404 | 0.649 | 0.058 |
| LRCM9-like | 0.567 | 0.143 | -0.242 | 0.564 | -0.570 | 0.109 | 0.418 | 0.263 | -0.163 | 0.699 |
| nAChR-like subunit | 0.223 | 0.564 | -0.209 | 0.589 | -0.127 | 0.726 | 0.097 | 0.790 | 0.010 | 0.979 |
| Peroxinectin-like | 0.401 | 0.285 | -0.035 | 0.928 | 0.225 | 0.531 | 0.581 | 0.078 | 0.542 | 0.132 |
| TPAP | -0.013 | 0.977 | 0.291 | 0.526 | 0.228 | 0.622 | -0.354 | 0.435 | 0.282 | 0.588 |
| Trypsin 1 | 0.089 | 0.834 | 0.489 | 0.218 | 0.371 | 0.325 | -0.415 | 0.267 | -0.062 | 0.884 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult male *Lepeophtheirus salmonis* genes and the MNRQ of five markers of Atlantic salmon immune response in spleen tissue on day 14 of a 14 day cohabitation of Atlantic salmon initially infected with *L. salmonis*.  CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table B-9** Pearson correlations between the expression of salmon immune markers in salmon spleen in initially uninfected fish and expression of 8 genes in male *Lepeophtheirus salmonis* on day 2 of a 14 day cohabitation study.

Male Lice vs. Spleen: New Host 2dpc

| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value |
| CYP18 A1-like | -0.378 | 0.282 | -0.374 | 0.409 | 0.240 | 0.504 | 0.100 | 0.784 | -0.436 | 0.241 |
| CYP450 I-1 like | 0.174 | 0.742 | 0.332 | 0.586 | -0.093 | 0.861 | 0.161 | 0.761 | 0.525 | 0.364 |
| GRα-2-like | -0.134 | 0.711 | 0.199 | 0.668 | -0.114 | 0.754 | 0.287 | 0.422 | 0.743 | 0.022 |
| LRCM9-like | 0.083 | 0.819 | -0.181 | 0.698 | 0.196 | 0.588 | 0.102 | 0.778 | -0.119 | 0.760 |
| nAChR-like subunit | 0.104 | 0.774 | -0.061 | 0.896 | 0.230 | 0.522 | 0.237 | 0.510 | 0.009 | 0.981 |
| Peroxinectin-like | -0.531 | 0.141 | -0.199 | 0.706 | -0.552 | 0.123 | -0.534 | 0.139 | 0.325 | 0.433 |
| TPAP | 0.227 | 0.557 | -0.044 | 0.934 | -0.278 | 0.469 | -0.104 | 0.790 | -0.535 | 0.172 |
| Trypsin 1 | -0.144 | 0.711 | -0.016 | 0.972 | 0.756 | 0.018 | -0.213 | 0.583 | -0.554 | 0.154 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult male *Lepeophtheirus salmonis* genes and the MNRQ of five markers of Atlantic salmon immune response in spleen tissue on day 2 of a 14 day cohabitation of Atlantic salmon initially uninfected with *L. salmonis*. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with $p>0.05$ are significantly different than 0.

**Table B-10** Pearson correlations between the expression of salmon immune markers in salmon spleen in initially uninfected fish and expression of 8 genes in male *Lepeophtheirus salmonis* on day 14 of a 14 day cohabitation study.

Male Lice vs. Spleen: New Host 14dpc

| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value | Pearson C | P value |
| CYP18 A1-like | 0.270 | 0.451 | -0.181 | 0.616 | -0.034 | 0.926 | 0.454 | 0.187 | 0.333 | 0.382 |
| CYP450 I-1 like | -0.459 | 0.360 | 0.110 | 0.835 | -0.518 | 0.293 | 0.038 | 0.943 | -0.470 | 0.347 |
| GRα-2-like | -0.381 | 0.312 | -0.309 | 0.419 | -0.525 | 0.147 | -0.327 | 0.391 | 0.147 | 0.729 |
| LRCM9-like | 0.033 | 0.932 | 0.481 | 0.190 | 0.403 | 0.282 | 0.349 | 0.357 | -0.194 | 0.646 |
| nAChR-like subunit | 0.216 | 0.550 | 0.225 | 0.531 | 0.033 | 0.929 | 0.213 | 0.555 | -0.524 | 0.148 |
| Peroxinectin-like | 0.216 | 0.549 | -0.153 | 0.673 | -0.234 | 0.516 | -0.448 | 0.194 | -0.551 | 0.124 |
| TPAP | -0.613 | 0.144 | 0.409 | 0.362 | -0.020 | 0.966 | -0.653 | 0.112 | -0.059 | 0.911 |
| Trypsin 1 | -0.314 | 0.377 | -0.262 | 0.464 | -0.214 | 0.553 | 0.244 | 0.496 | 0.303 | 0.429[i] |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult male *Lepeophtheirus salmonis* genes and the MNRQ of five markers of Atlantic salmon immune response in spleen tissue on day 14 of a 14 day cohabitation of Atlantic salmon initially uninfected with *L. salmonis*. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with $p>0.05$ are significantly different than 0.

**Table B-11** Pearson correlations between the expression of salmon immune markers in salmon skin and expression of 8 genes in female *Lepeophtheirus salmonis* across all time points of a 14 day cohabitation study.

Female Lice vs. Skin: All time points

| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | p value | Pearson C | p value | Pearson C | p value | Pearson C | p value | Pearson C | p value |
| CYP18 A1-like | 0.184 | 0.452 | -0.044 | 0.858 | 0.398 | 0.092 | 0.455 | 0.057 | 0.009 | 0.971 |
| CYP450 I-1 like | -0.232 | 0.406 | -0.375 | 0.168 | -0.434 | 0.106 | -0.167 | 0.568 | -0.492 | 0.062 |
| GRα-2-like | 0.027 | 0.917 | -0.101 | 0.700 | 0.264 | 0.306 | 0.157 | 0.561 | -0.255 | 0.323 |
| LRCM9-like | -0.161 | 0.536 | -0.110 | 0.674 | 0.144 | 0.582 | 0.081 | 0.767 | -0.191 | 0.462 |
| nAChR-like subunit | -0.233 | 0.336 | -0.143 | 0.559 | -0.087 | 0.724 | -0.252 | 0.312 | -0.160 | 0.514 |
| Peroxinectin-like | 0.225 | 0.370 | 0.265 | 0.289 | 0.587 | 0.010 | 0.638 | 0.006 | 0.417 | 0.085 |
| TPAP | 0.042 | 0.882 | 0.016 | 0.956 | 0.186 | 0.507 | -0.140 | 0.633 | -0.012 | 0.966 |
| Trypsin 1 | 0.217 | 0.387 | 0.041 | 0.872 | 0.392 | 0.107 | 0.365 | 0.150 | -0.266 | 0.285 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult female *Lepeophtheirus salmonis* genes and the MNRQ of five markers of Atlantic salmon immune response in skin tissue across all time points of a 14 day cohabitation of Atlantic salmon initially infected and uninfected with *L. salmonis*. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with $p>0.05$ are significantly different than 0.

**Table B-12** Pearson correlations between the expression of salmon immune markers in salmon skin in initially infected fish and expression of 8 genes in female *Lepeophtheirus salmonis* on day 2 of a 14 day cohabitation study.

Female Lice vs. Skin: Initial Host 2dpc

| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | p value | Pearson C | p value | Pearson C | p value | Pearson C | p value | Pearson C | p value |
| CYP18 A1-like | -0.172 | 0.635 | -0.027 | 0.942 | 0.093 | 0.799 | 0.154 | 0.672 | 0.032 | 0.930 |
| CYP450 I-1 like | -0.642 | 0.086 | -0.615 | 0.105 | -0.615 | 0.105 | -0.430 | 0.288 | -0.558 | 0.151 |
| GRα-2-like | -0.310 | 0.417 | -0.328 | 0.389 | -0.249 | 0.519 | -0.259 | 0.502 | -0.316 | 0.407 |
| LRCM9-like | -0.120 | 0.757 | -0.209 | 0.589 | 0.173 | 0.656 | -0.313 | 0.412 | -0.267 | 0.488 |
| nAChR-like subunit | 0.043 | 0.907 | -0.121 | 0.740 | 0.273 | 0.446 | -0.326 | 0.359 | -0.211 | 0.558 |
| Peroxinectin-like | 0.434 | 0.243 | 0.630 | 0.069 | 0.676 | 0.046 | 0.681 | 0.043 | 0.667 | 0.050 |
| TPAP | 0.153 | 0.717 | -0.035 | 0.934 | 0.244 | 0.561 | -0.213 | 0.612 | -0.113 | 0.790 |
| Trypsin 1 | -0.700 | 0.036 | -0.381 | 0.311 | -0.336 | 0.377 | 0.165 | 0.672 | -0.200 | 0.606 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult female *Lepeophtheirus salmonis* genes and the MNRQ of five markers of Atlantic salmon immune response in skin tissue on day 2 of a 14 day cohabitation of Atlantic salmon initially infected with *L. salmonis*. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table B-13** Pearson correlations between the expression of salmon immune markers in salmon skin in initially infected fish and expression of 8 genes in female *Lepeophtheirus salmonis* on day 14 of a 14 day cohabitation study.

Female Lice vs. Skin: Initial Host 14dpc

| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | p value | Pearson C | p value | Pearson C | p value | Pearson C | p value | Pearson C | p value |
| CYP18 A1-like | 0.364 | 0.335 | -0.010 | 0.980 | 0.657 | 0.054 | 0.652 | 0.080 | 0.134 | 0.731 |
| CYP450 I-1 like | -0.004 | 0.993 | 0.216 | 0.642 | -0.160 | 0.732 | 0.012 | 0.982 | -0.377 | 0.405 |
| GRα-2-like | 0.347 | 0.400 | 0.387 | 0.344 | 0.760 | 0.029 | 0.447 | 0.315 | 0.049 | 0.908 |
| LRCM9-like | -0.176 | 0.677 | 0.182 | 0.666 | 0.074 | 0.862 | 0.219 | 0.637 | 0.191 | 0.651 |
| nAChR-like subunit | -0.499 | 0.172 | -0.145 | 0.709 | -0.648 | 0.059 | -0.304 | 0.464 | 0.092 | 0.814 |
| Peroxinectin-like | 0.087 | 0.824 | -0.093 | 0.813 | 0.476 | 0.195 | 0.569 | 0.141 | 0.398 | 0.289 |
| TPAP | 0.085 | 0.856 | -0.278 | 0.546 | 0.343 | 0.451 | 0.751 | 0.085 | 0.339 | 0.456 |
| Trypsin 1 | 0.577 | 0.104 | 0.592 | 0.093 | 0.828 | 0.006 | 0.264 | 0.528 | -0.305 | 0.424 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult female *Lepeophtheirus salmonis* genes and the MNRQ of five markers of Atlantic salmon immune response in skin tissue on day 14 of a 14 day cohabitation of Atlantic salmon initially infected with *L. salmonis*. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed in *L. salmonis*. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table B-14** Pearson correlations between the expression of salmon immune markers in salmon spleen and expression of 8 genes in female *Lepeophtheirus salmonis* across all time points of a 14 day cohabitation study.

Female Lice vs. Spleen: All time points

| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | p value | Pearson C | p value | Pearson C | p value | Pearson C | p value | Pearson C | p value |
| CYP18 A1-like | 0.014 | 0.956 | -0.235 | 0.333 | 0.281 | 0.244 | -0.144 | 0.556 | -0.043 | 0.862 |
| CYP450 I-1 like | -0.551 | 0.033 | -0.024 | 0.932 | 0.305 | 0.268 | -0.395 | 0.145 | 0.283 | 0.307 |
| GRα-2-like | -0.226 | 0.382 | -0.217 | 0.403 | 0.232 | 0.371 | -0.363 | 0.152 | -0.344 | 0.176 |
| LRCM9-like | -0.180 | 0.489 | 0.243 | 0.347 | 0.387 | 0.125 | -0.205 | 0.430 | 0.049 | 0.852 |
| nAChR-like subunit | -0.082 | 0.739 | 0.451 | 0.052 | 0.175 | 0.473 | -0.065 | 0.791 | 0.230 | 0.343 |
| Peroxinectin-like | 0.457 | 0.057 | 0.088 | 0.727 | 0.147 | 0.560 | 0.447 | 0.063 | -0.039 | 0.879 |
| TPAP | 0.072 | 0.799 | 0.140 | 0.619 | -0.028 | 0.922 | -0.147 | 0.602 | -0.012 | 0.965 |
| Trypsin 1 | -0.118 | 0.640 | -0.465 | 0.052 | 0.244 | 0.329 | -0.453 | 0.059 | -0.361 | 0.141 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult female *Lepeophtheirus salmonis* genes and the MNRQ of five markers of Atlantic salmon immune response in spleen tissue across all time points of a 14 day cohabitation of Atlantic salmon initially infected and uninfected with *L. salmonis*. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table B-15** Pearson correlations between the expression of salmon immune markers in salmon spleen in initially infected fish and expression of 8 genes in female *Lepeophtheirus salmonis* on day 2 of a 14 day cohabitation study.

Female Lice vs. Spleen: Initial Host 2dpc

| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | p value | Pearson C | p value | Pearson C | p value | Pearson C | p value | Pearson C | p value |
| CYP18 A1-like | 0.084 | 0.817 | -0.150 | 0.679 | 0.037 | 0.920 | 0.037 | 0.920 | 0.200 | 0.579 |
| CYP450 I-1 like | -0.647 | 0.083 | -0.465 | 0.246 | 0.597 | 0.118 | -0.452 | 0.261 | -0.495 | 0.212 |
| GRα-2-like | -0.288 | 0.453 | -0.250 | 0.516 | 0.329 | 0.388 | -0.316 | 0.408 | -0.222 | 0.567 |
| LRCM9-like | -0.019 | 0.962 | 0.203 | 0.601 | 0.238 | 0.538 | -0.347 | 0.360 | -0.216 | 0.576 |
| nAChR-like subunit | 0.047 | 0.898 | 0.444 | 0.198 | 0.153 | 0.673 | -0.313 | 0.379 | -0.253 | 0.481 |
| Peroxinectin-like | 0.687 | 0.041 | 0.219 | 0.571 | -0.621 | 0.075 | 0.668 | 0.049 | 0.657 | 0.054 |
| TPAP | 0.083 | 0.845 | 0.410 | 0.313 | 0.064 | 0.880 | -0.199 | 0.637 | -0.169 | 0.690 |
| Trypsin 1 | -0.278 | 0.469 | -0.791 | 0.011 | 0.371 | 0.326 | -0.093 | 0.812 | 0.211 | 0.586 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult female *Lepeophtheirus salmonis* genes and the MNRQ of five markers of Atlantic salmon immune response in spleen tissue on day 2 of a 14 day cohabitation of Atlantic salmon initially infected with *L. salmonis*.  CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table B-16** Pearson correlations between the expression of salmon immune markers in salmon spleen in initially infected fish and expression of 8 genes in female *Lepeophtheirus salmonis* on day 14 of a 14 day cohabitation study.

Female Lice vs. Spleen: Initial Host 14dpc

| | IL-1β | | IL-8 | | IL-12 | | IgT | | MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | p value | Pearson C | p value | Pearson C | p value | Pearson C | p value | Pearson C | p value |
| CYP18 A1-like | -0.041 | 0.917 | -0.320 | 0.401 | 0.374 | 0.322 | -0.295 | 0.441 | -0.102 | 0.793 |
| CYP450 I-1 like | -0.339 | 0.456 | 0.153 | 0.743 | 0.427 | 0.340 | -0.618 | 0.139 | 0.670 | 0.100 |
| GRα-2-like | -0.100 | 0.815 | -0.379 | 0.355 | 0.228 | 0.587 | -0.435 | 0.282 | -0.498 | 0.210 |
| LRCM9-like | -0.472 | 0.237 | 0.239 | 0.568 | 0.553 | 0.155 | -0.141 | 0.739 | 0.112 | 0.792 |
| nAChR-like subunit | -0.371 | 0.326 | 0.564 | 0.114 | 0.240 | 0.533 | 0.279 | 0.467 | 0.503 | 0.168 |
| Peroxinectin-like | -0.006 | 0.988 | -0.125 | 0.749 | 0.210 | 0.588 | 0.120 | 0.759 | -0.430 | 0.248 |
| TPAP | -0.334 | 0.464 | 0.060 | 0.898 | 0.713 | 0.072 | -0.064 | 0.892 | 0.328 | 0.472 |
| Trypsin 1 | 0.075 | 0.848 | -0.608 | 0.082 | 0.001 | 0.998 | -0.745 | 0.021 | -0.478 | 0.193 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult female *Lepeophtheirus salmonis* genes and the MNRQ of five markers of Atlantic salmon immune response in spleen tissue on day 14 of a 14 day cohabitation of Atlantic salmon initially infected with *L. salmonis*. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

## Appendix C - Pearson correlations of *Lepeophtheirus salmonis* and Atlantic salmon gene expression during high or low infection levels

**Table C-1** Pearson correlations between the expression of salmon immune markers in salmon skin in fish with a high infection level and expression of 8 genes in male *Lepeophtheirus salmonis* during a 14 day cohabitation study.

High Male Infection vs. Skin

| | Skin IL-1β | | Skin IL-8 | | Skin IL-12 | | Skin IgT | | Skin MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue |
| CYP18 A1-like | 0.716 | 0.070 | 0.758 | 0.048 | 0.794 | 0.033 | -0.051 | 0.914 | 0.519 | 0.233 |
| CYP450 I-1 like | -0.112 | 0.833 | 0.366 | 0.476 | 0.487 | 0.327 | 0.349 | 0.498 | 0.303 | 0.560 |
| GRα-2-like | -0.546 | 0.205 | -0.814 | 0.026 | -0.731 | 0.062 | -0.279 | 0.544 | -0.251 | 0.588 |
| LRCM9-like | -0.509 | 0.243 | -0.530 | 0.221 | -0.544 | 0.207 | -0.003 | 0.996 | -0.500 | 0.253 |
| nAChR-like subunit | -0.298 | 0.517 | -0.613 | 0.143 | -0.561 | 0.190 | -0.369 | 0.415 | -0.292 | 0.525 |
| Peroxinectin-like | -0.542 | 0.208 | -0.774 | 0.041 | -0.708 | 0.075 | -0.239 | 0.606 | -0.307 | 0.504 |
| TPAP | -0.965 | 0.002 | -0.504 | 0.308 | -0.465 | 0.352 | 0.763 | 0.077 | -0.275 | 0.598 |
| Trypsin 1 | -0.058 | 0.902 | 0.281 | 0.541 | 0.314 | 0.493 | 0.322 | 0.481 | 0.024 | 0.958 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult male *Lepeophtheirus salmonis* peroxinectin-like gene expression and the MNRQ of five markers of Atlantic salmon immune response in skin tissue of salmon with a high infection level. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, Peroxinectin-like, and Trypsin-1 were analysed Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table C-2** Pearson correlations between the expression of salmon immune markers in salmon skin in fish with a low infection level and expression of 8 genes in male *Lepeophtheirus salmonis* during a 14 day cohabitation study.

Low Male Infection vs. Skin

| | Skin IL-1β | | Skin IL-8 | | Skin IL-12 | | Skin IgT | | Skin MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue |
| CYP18 A1-like | -0.243 | 0.643 | 0.048 | 0.929 | -0.905 | 0.013 | -0.746 | 0.089 | 0.438 | 0.385 |
| CYP450 I-1 like | -0.054 | 0.920 | 0.584 | 0.224 | 0.259 | 0.621 | -0.059 | 0.912 | 0.693 | 0.127 |
| GRα-2-like | 0.218 | 0.678 | 0.264 | 0.613 | 0.845 | 0.034 | 0.575 | 0.233 | 0.329 | 0.524 |
| LRCM9-like | 0.319 | 0.537 | -0.192 | 0.716 | 0.901 | 0.014 | 0.819 | 0.046 | -0.557 | 0.251 |
| nAChR-like subunit | 0.095 | 0.859 | 0.150 | 0.777 | 0.875 | 0.023 | 0.787 | 0.063 | 0.120 | 0.821 |
| peroxinectin-like | 0.018 | 0.972 | -0.220 | 0.675 | 0.813 | 0.049 | 0.918 | 0.010 | -0.132 | 0.803 |
| TPAP | -0.143 | 0.818 | 0.128 | 0.838 | 0.586 | 0.299 | 0.677 | 0.210 | -0.025 | 0.968 |
| Trypsin 1 | -0.213 | 0.685 | 0.559 | 0.249 | -0.249 | 0.634 | -0.260 | 0.619 | 0.008 | 0.988 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult male *Lepeophtheirus salmonis* peroxinectin-like gene expression and the MNRQ of five markers of Atlantic salmon immune response in skin tissue of salmon with a low infection level. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table C-3** Pearson correlations between the expression of salmon immune markers in salmon spleen in fish with a high infection level and expression of 8 genes in male *Lepeophtheirus salmonis* during a 14 day cohabitation study.

High Male Infection vs. Spleen

| | Skin IL-1β | | Skin IL-8 | | Skin IL-12 | | Skin IgT | | Skin MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue |
| CYP18 A1-like | 0.823 | 0.023 | 0.252 | 0.586 | -0.244 | 0.599 | -0.096 | 0.837 | 0.061 | 0.897 |
| CYP450 I-1 like | 0.199 | 0.706 | 0.547 | 0.262 | 0.334 | 0.517 | 0.482 | 0.333 | 0.509 | 0.302 |
| GRα-2-like | -0.586 | 0.166 | -0.318 | 0.487 | -0.087 | 0.853 | -0.096 | 0.838 | -0.127 | 0.787 |
| LRCM9-like | -0.702 | 0.079 | -0.412 | 0.358 | 0.103 | 0.827 | 0.046 | 0.922 | 0.069 | 0.884 |
| nAChR-like subunit | -0.469 | 0.288 | -0.525 | 0.226 | -0.256 | 0.580 | -0.245 | 0.597 | -0.161 | 0.730 |
| Peroxinectin-like | -0.619 | 0.138 | -0.354 | 0.436 | -0.061 | 0.896 | -0.079 | 0.866 | -0.095 | 0.839 |
| TPAP | -0.799 | 0.057 | 0.611 | 0.198 | 0.896 | 0.016 | 0.811 | 0.050 | 0.543 | 0.265 |
| Trypsin 1 | -0.003 | 0.995 | 0.180 | 0.699 | 0.253 | 0.584 | 0.341 | 0.454 | 0.441 | 0.322 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult male *Lepeophtheirus salmonis* peroxinectin-like gene expression and the MNRQ of five markers of Atlantic salmon immune response in spleen tissue of salmon with a high infection level. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with $p>0.05$ are significantly different than 0.

**Table C-4** Pearson correlations between the expression of salmon immune markers in salmon spleen in fish with a low infection level and expression of 8 genes in male *Lepeophtheirus salmonis* during a 14 day cohabitation study.

| Low Male Infection vs. Spleen | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Skin IL-1β | | Skin IL-8 | | Skin IL-12 | | Skin IgT | | Skin MMP9 | |
| | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue |
| CYP18 A1-like | 0.057 | 0.914 | 0.386 | 0.449 | -0.454 | 0.365 | 0.115 | 0.828 | 0.142 | 0.788 |
| CYP450 I-1 like | 0.616 | 0.193 | 0.053 | 0.920 | -0.303 | 0.559 | 0.637 | 0.173 | 0.902 | 0.014 |
| GRα-2-like | 0.366 | 0.476 | 0.149 | 0.778 | 0.154 | 0.770 | -0.038 | 0.943 | 0.506 | 0.306 |
| LRCM9-like | -0.236 | 0.652 | -0.253 | 0.628 | 0.574 | 0.233 | -0.410 | 0.419 | -0.368 | 0.473 |
| nAChR-like subunit | 0.028 | 0.958 | 0.078 | 0.883 | 0.493 | 0.321 | -0.294 | 0.572 | 0.224 | 0.669 |
| Peroxinectin-like | -0.122 | 0.818 | 0.028 | 0.958 | 0.619 | 0.190 | -0.651 | 0.162 | -0.106 | 0.842 |
| TPAP | -0.641 | 0.244 | 0.263 | 0.669 | 0.847 | 0.070 | -0.680 | 0.206 | -0.235 | 0.704 |
| Trypsin 1 | -0.387 | 0.449 | -0.254 | 0.627 | 0.185 | 0.725 | 0.574 | 0.234 | 0.052 | 0.922 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult male *Lepeophtheirus salmonis* peroxinectin-like gene expression and the MNRQ of five markers of Atlantic salmon immune response in spleen tissue of salmon with a low infection level. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table C-5** Pearson correlations between the expression of salmon immune markers in salmon skin in fish with a high infection level and expression of 8 genes in female *Lepeophtheirus salmonis* during a 14 day cohabitation study.

High Female infection vs. Skin

| | Skin IL-1β | | Skin IL-8 | | Skin IL-12 | | Skin IgT | | Skin MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue |
| CYP18 A1-like | 0.174 | 0.569 | 0.091 | 0.767 | 0.059 | 0.848 | -0.191 | 0.532 | -0.125 | 0.684 |
| CYP450 I-1 like | -0.286 | 0.395 | -0.481 | 0.134 | -0.482 | 0.133 | -0.010 | 0.976 | -0.466 | 0.149 |
| GRα-2-like | 0.217 | 0.476 | 0.324 | 0.281 | 0.488 | 0.091 | 0.023 | 0.939 | 0.441 | 0.131 |
| LRCM9-like | 0.362 | 0.225 | 0.465 | 0.109 | 0.344 | 0.249 | 0.088 | 0.774 | -0.144 | 0.638 |
| nAChR-like subunit | -0.107 | 0.729 | 0.026 | 0.932 | 0.235 | 0.439 | 0.171 | 0.578 | 0.537 | 0.059 |
| Peroxinectin-like | 0.420 | 0.153 | 0.537 | 0.058 | 0.836 | 0.000 | -0.095 | 0.759 | 0.739 | 0.004 |
| TPAP | 0.005 | 0.988 | -0.097 | 0.764 | 0.017 | 0.957 | -0.241 | 0.451 | -0.235 | 0.439 |
| Trypsin 1 | 0.465 | 0.110 | 0.349 | 0.243 | 0.041 | 0.894 | 0.154 | 0.632 | -0.707 | 0.007 |

Pears Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult female *Lepeophtheirus salmonis* peroxinectin-like gene expression and the MNRQ of five markers of Atlantic salmon immune response in skin tissue of salmon with a high infection level. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with $p>0.05$ are significantly different than 0.

**Table C-6** Pearson correlations between the expression of salmon immune markers in salmon skin in fish with a low infection level and expression of 8 genes in female *Lepeophtheirus salmonis* during a 14 day cohabitation study.

Low Female infection vs. Skin

| | Skin IL-1β | | Skin IL-8 | | Skin IL-12 | | Skin IgT | | Skin MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue |
| CYP18 A1-like | -0.404 | 0.427 | -0.233 | 0.657 | 0.780 | 0.067 | 0.889 | 0.043 | -0.227 | 0.666 |
| CYP450 I-1 like | 0.574 | 0.426 | -0.422 | 0.578 | -0.261 | 0.739 | 1.000 | 0.010 | -0.614 | 0.386 |
| GRα-2-like | -0.228 | 0.663 | -0.335 | 0.516 | 0.820 | 0.046 | 0.926 | 0.024 | -0.323 | 0.532 |
| LRCM9-like | -0.436 | 0.388 | 0.411 | 0.418 | 0.215 | 0.682 | 0.005 | 0.994 | 0.482 | 0.333 |
| nAChR-like subunit | 0.342 | 0.506 | -0.272 | 0.602 | -0.640 | 0.171 | -0.498 | 0.393 | -0.410 | 0.419 |
| Peroxinectin-like | -0.528 | 0.281 | 0.177 | 0.738 | 0.713 | 0.112 | 0.551 | 0.336 | 0.305 | 0.556 |
| TPAP | 0.196 | 0.752 | -0.214 | 0.730 | 0.195 | 0.753 | 0.527 | 0.473 | -0.288 | 0.638 |
| Trypsin 1 | -0.508 | 0.304 | -0.269 | 0.606 | 0.899 | 0.015 | 0.969 | 0.007 | -0.215 | 0.683 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult female *Lepeophtheirus salmonis* peroxinectin-like gene expression and the MNRQ of five markers of Atlantic salmon immune response in skin tissue of salmon with a low infection level. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table C-7** Pearson correlations between the expression of salmon immune markers in salmon spleen in fish with a high infection level and expression of 8 genes in female *Lepeophtheirus salmonis* during a 14 day cohabitation study.

High Female infection vs. Spleen

| | Skin IL-1β | | Skin IL-8 | | Skin IL-12 | | Skin IgT | | Skin MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue |
| CYP18 A1-like | 0.047 | 0.880 | -0.210 | 0.490 | -0.208 | 0.495 | -0.218 | 0.474 | -0.180 | 0.557 |
| CYP450 I-1 like | -0.482 | 0.133 | -0.201 | 0.553 | 0.047 | 0.890 | -0.032 | 0.925 | -0.144 | 0.672 |
| GRα-2-like | 0.521 | 0.068 | 0.229 | 0.452 | -0.038 | 0.902 | 0.049 | 0.872 | 0.099 | 0.749 |
| LRCM9-like | 0.289 | 0.338 | -0.075 | 0.809 | -0.048 | 0.876 | -0.196 | 0.521 | -0.362 | 0.224 |
| nAChR-like subunit | 0.293 | 0.331 | 0.416 | 0.157 | 0.182 | 0.552 | 0.315 | 0.295 | 0.376 | 0.205 |
| Peroxinectin-like | 0.898 | 0.000 | 0.293 | 0.332 | -0.187 | 0.541 | -0.007 | 0.983 | 0.139 | 0.651 |
| TPAP | 0.055 | 0.866 | -0.057 | 0.861 | -0.188 | 0.558 | -0.054 | 0.867 | 0.118 | 0.716 |
| Trypsin 1 | -0.061 | 0.842 | -0.599 | 0.030 | -0.350 | 0.241 | -0.587 | 0.035 | -0.733 | 0.004 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult female *Lepeophtheirus salmonis* peroxinectin-like gene expression and the MNRQ of five markers of Atlantic salmon immune response in spleen tissue of salmon with a high infection level. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

**Table C-8** Pearson correlations between the expression of salmon immune markers in salmon spleen in fish with a low infection level and expression of 8 genes in female *Lepeophtheirus salmonis* during a 14 day cohabitation study.

Low Female infection vs. Spleen

| | Skin IL-1β | | Skin IL-8 | | Skin IL-12 | | Skin IgT | | Skin MMP9 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue | Pearson C | Pvalue |
| CYP18 A1-like | -0.487 | 0.328 | -0.148 | 0.780 | 0.849 | 0.033 | -0.476 | 0.340 | -0.167 | 0.752 |
| CYP450 I-1 like | -0.735 | 0.265 | 0.587 | 0.413 | 0.653 | 0.347 | -0.939 | 0.061 | 0.663 | 0.337 |
| GRα-2-like | -0.608 | 0.200 | -0.215 | 0.682 | 0.846 | 0.034 | -0.456 | 0.363 | -0.506 | 0.306 |
| LRCM9-like | 0.438 | 0.385 | 0.002 | 0.998 | -0.177 | 0.738 | 0.382 | 0.454 | 0.112 | 0.832 |
| nAChR-like subunit | -0.261 | 0.617 | 0.269 | 0.607 | 0.080 | 0.881 | -0.529 | 0.281 | 0.762 | 0.078 |
| Peroxinectin-like | 0.195 | 0.711 | -0.293 | 0.573 | 0.140 | 0.791 | 0.307 | 0.554 | -0.468 | 0.349 |
| TPAP | -0.531 | 0.357 | 0.255 | 0.678 | 0.554 | 0.332 | -0.492 | 0.400 | 0.064 | 0.919 |
| Trypsin 1 | -0.402 | 0.430 | -0.309 | 0.551 | 0.814 | 0.048 | -0.381 | 0.456 | -0.310 | 0.550 |

Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult female *Lepeophtheirus salmonis* peroxinectin-like gene expression and the MNRQ of five markers of Atlantic salmon immune response in spleen tissue of salmon with a low infection level. CYP18 A1-like, Cytochrome p450 Isoform 1-like Protein (CYP450 I-1), glycene receptor α-2 Like (GRα-2), leukocyte receptor cluster member 9 (LCRM9), nicotinic acetylcholine receptor subunit (nAChR-like subunit), tissue plasminogen activator precursor (TPAP)-like, peroxinectin-like, and Trypsin-1 were analysed Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with p>0.05 are significantly different than 0.

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